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THE HYDROPHOBIC EFFECT: FORMATION OF MICELLES AND BIOLOGICAL MEMBRANES

Second Edition

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A WILEY-INTERSCIENCE PUBLICATION

JOHN WILEY & SONS, New York • Chichester • Brisbane • Toronto

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Library of Congress Cataloging in Publication Data:

Tanford, Charles, 1921–
The hydrophobic effect.

"A Wiley-Interscience publication."
Includes bibliographies and indexes.
1. Membranes (Biology) 2. Micelles. 3. Surface chemistry. 4. Solution (Chemistry) I. Title.

QH601.T36 1979 574.1'9283 79-13597
ISBN 0-471-04893-3

Printed in the United States of America

10 9 8 7 6 5

PREFACE TO THE SECOND EDITION

> *However hard we try to bring in the new,*
> *it comes into being only in the midst of*
> *clumsy deals.*
>
> PETER WEISS, *Marat/Sade*

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In the first edition of this book I attempted to give a comprehensive account of the hydrophobic effect and of its ability to act as an organizing force in the formation of surface films, micelles, lipid bilayers, protein–amphiphile complexes, and biological membranes. Being a first attempt, the book had many shortcomings, attributable to the fact that I initially perceived some of the unifying principles only clumsily and in some instances incorrectly. A strong personal motivation for writing the second edition is to correct those parts of the text where (hopefully) I can now present a conceptually clearer or theoretically more rigorous treatment.

The chapters on micelle formation contain the most important example: it has been known for a long time that the existence of micelles depends on the existence of opposing forces, but mathematical analysis shows that not only must the two major contributions to the free energy of micellization have opposite signs, but also *their first derivatives with respect to micelle size.* I did not recognize the physical origin of the negative first derivative of the hydrophobic free energy contribution until after the first edition was published, so that it is now possible to present a much more satisfying account of the phenomenon of micellization. These chapters have therefore been almost completely rewritten, and they demonstrate that one can in principle (and semiquantitatively in practice) predict the optimal size and size distribution of micelles formed by simple amphiphiles, as well as the critical concentration at which micelles are first formed. Another important area of improved understanding concerns the combination of proteins with amphiphiles, where, for example, it is now possible to give a plausible theoretical explanation for why ionic detergents such as sodium dodecyl sulfate tend to denature proteins, whereas nonionic detergents often do not do so.

A second motivation for writing a new edition (perhaps more appealing to many readers than my first one) is that nearly all aspects of the broad field covered by this book are the subject of intensive research, so that there has been a spectacular growth in factual knowledge about structure and function, and in the techniques available to study them. This is true especially for the topics most relevant to biology: the properties of phospholipid bilayers, and the identification and characterization of membrane proteins. I have attempted, within the limitations imposed by the length of this book, to incorporate most of the new developments that have taken place; almost half the literature references in this edition refer to work done since 1973.

In conclusion, I want to acknowledge my indebtedness to my colleague, Dr. Jacqueline A. Reynolds, who has critically read much of the text of this edition and who has been a constant source of invaluable advice and information. The chapter on serum lipoproteins (which is entirely new) was written in collaboration with her, and it includes a summary of her recent research focusing on the difference between the modes of protein–lipid interaction that lead to *soluble* lipoproteins on the one hand and *membrane-bound* proteins on the other. I also wish to express my appreciation for continued research support from the National Science Foundation and the National Institutes of Health.

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Durham, North Carolina
July 1979

PREFACE TO THE FIRST EDITION

The hydrophobic effect is perhaps the most important single factor in the organization of the constituent molecules of living matter into complex structural entities such as cell membranes and organelles. It is equally important in the formation of detergent micelles and other phenomena that occur in aqueous solution. In spite of this, no comprehensive account of the hydrophobic effect exists, and this book is intended to fill that gap. It begins with the simplest manifestation of the hydrophobic effect—the meager solubility of hydrocarbons in water—and continues with more complex phenomena, such as the formation of micelles, surface films, and lipid bilayers, and the interaction of proteins with hydrophobic ligands. The final chapters relate these topics to the structural and functional properties of biological membranes, which are as yet only poorly understood and the subject of much current research.

In discussing these topics the emphasis is on experimental results and on deductions that can be rigorously derived from them. The underlying thermodynamic principles are presented in some detail, but speculative models that are not directly supported by experimental data are not discussed. A chapter on the structure of water is included, since therein must lie the basis for the hydrophobic effect; here too, however, the focus is on experiment rather than on theoretical models.

I am indebted to numerous colleagues who have helped to clarify my ideas about the subject matter of this book: in particular to Professors J. A. Reynolds and W. Kauzmann, for their valuable suggestions concerning several parts of the manuscript; to Dr. R. Smith, who carried out several technically difficult experimental studies; and to Professor J. D. Robertson, who helped initiate me into the membrane field. I also wish to express my appreciation to the National Science Foundation and to the National Institutes of Health for their support of my research, some of the results of which form an integral part of this book.

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Durham, North Carolina
March 1973

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"The antipathy of the paraffin-chain for water is, however, frequently misunderstood. There is no question of actual repulsion between individual water molecules and paraffin chains, nor is there any very strong attraction of paraffin chains for one another. There is, however, a very strong attraction of water molecules for one another in comparison with which the paraffin-paraffin or paraffin-water attractions are very slight."

G. S. HARTLEY (1936)

"Possibly the decisive step (in the origin of life) was the formation of the first cell, in which chain molecules of at least two of three types now represented by nucleic acids, proteins, and polysaccharides were enclosed in a semi-permeable membrane which kept them together but let their food in."

J. B. S. HALDANE (1954)

THE HYDROPHOBIC EFFECT: FORMATION OF MICELLES AND BIOLOGICAL MEMBRANES

ONE

INTRODUCTION

Life as we know it originated in water and could not exist in its absence. The importance of water's unique physical properties and of its unique solvent power, especially for ions, has been frequently cited (e.g., Henderson, 1913), but the equally great importance of its unique lack of solvent power for many nonpolar substances has received far less attention. That is what this book is about: molecules that are soluble in alcohol, ether, and many other solvents, but not in water; and molecules of a dual nature, with one part soluble in water and another part that is expelled from it. Molecules in the latter category are forced by their duality to adopt unique orientations with respect to the aqueous medium, and to form suitably organized structures. Such molecules are especially crucial to living matter in that they play a prominent role in its organization. The cell membrane, which in effect defines the living cell and allows it to exist as a coherent entity, is perhaps the best example. There can be little doubt that its formation is spontaneous, dependent only on the fact that its constituent molecules are partly hydrophilic and partly hydrophobic.

ORIGIN OF THE HYDROPHOBIC EFFECT

Hydrophobic substances are defined as substances that are readily soluble in many nonpolar solvents, but only sparingly soluble in water, distinct from substances that have generally low solubility in all solvents because they form solids with strong intermolecular cohesion. This distinction is probably especially important from the biological point of view, since it means that molecules expelled from water as a result of their hydrophobicity will tend to remain in a fluid, deformable state.

The existence of hydrophobic substances, and of organic molecules of a dual nature, containing hydrophobic portions, has been known for a long time, but misconceptions about the origin of the phenomenon have persisted until quite recently. Thus McBain, who can probably be credited with prime responsibility for demonstrating the reversible formation of micellar aggregates in aqueous soap solutions (McBain and Salmon, 1920), believed that the association between hydrocarbon chains in the formation of such micelles arises from "like to like" attraction, implying that the attraction of hydrocarbon chains for each other plays an important role in this process (McBain, 1950). Even Debye (1949) evidently shared this belief, since he equated the energy gained in micelle formation with the energy difference between liquid and gaseous hydrocarbon.

In fact, the attraction of nonpolar groups (such as hydrocarbon chains) for each other plays only a minor role in the hydrophobic effect. The effect actually arises primarily from the strong attractive forces between water molecules which, being isotropically arranged, must be disrupted or distorted when any solute is dissolved in the water. If the solute is ionic or strongly polar, it can form strong bonds to water molecules, which more than compensate for the disruption or distortion of the bonds existing in pure water; and ionic or polar substances thus tend to be easily soluble in water. No such compensation occurs with nonpolar groups, and their solution in water is accordingly resisted. In terms of the intermolecular bonds that are formed, the process of solution of a nonpolar solute S in water may be represented schematically as

$$2\,H_2O - H_2O + S \text{ — } S \rightleftharpoons 2\,H_2O \text{ — } S \text{ — } H_2O \qquad \text{(I)}$$

and the major factor driving the equilibrium in this reaction to the left is the strength of the hydrogen bonds between water molecules. These bonds are in fact so strong that the schematic representation of the product of reaction I as $H_2O \text{ — } S \text{ — } H_2O$ is probably misleading, since the hydrogen bonds tend to be maintained in distorted form, that is, $H_2\overline{O \text{ — } S \text{ — } H}_2O$ would be a better representation, at least at low temperatures (Frank and Evans, 1945). It is then a loss of entropy rather than bond energy that leads to an unfavorable free energy change for the process.

The true origin of the hydrophobic effect was qualitatively understood, or at least intuitively understood, by Traube (1891) on the basis of the phenomenon of surface activity. Traube showed that molecules consisting of a long hydrocarbon chain attached to a polar group (fatty acids, alcohols, amides, etc.), which have a measurable solubility in water by virtue of their constituent polar groups, tend to migrate to the surface of an aqueous solution, their presence there being recognized by a decrease in the surface tension. At very low concentrations of these molecules in the bulk solution, the reduction in surface tension increases linearly with the bulk concentration, but as the bulk concentration is raised it has progressively less effect. Traube recognized this as a saturation phenomenon, anticipating the more formal expression of this phenomenon by the adsorption equation of Langmuir (1917). The important aspect of the results in relation to the origin of the hydrophobic effect is that at low bulk concentrations, where the surface is sparsely populated and contacts between hydrocarbon chains are minimal, the ratio of concentration at the surface layer to that in the bulk medium increases approximately threefold for each CH_2 group added to the alkyl chain ("Traube's rule"). The same result was obtained for several homologous series involving different polar groups and, since attraction between alkyl groups cannot be important here, there is no ambiguity in the interpretation: the cause of the effect must reside solely in a lack of affinity between hydrocarbon and water. The same conclusion may be reached in another way. If attraction between hydrocarbon chains is important in migration to the surface, the process would become cooperative at low solute concentrations, and the surface concentration would not be linearly proportional to the concentration in bulk.

A direct way to demonstrate the dominant role of water self-attraction in the hydrocarbon-water system (based on bulk liquid rather than on molecular properties) is to employ the surface tensions of the pure liquids and the interfacial tension at a hydrocarbon-water interface (Tanford, 1979). These measurements yield the free energy gained when two portions of liquid, previously exposed to air, are brought into contact with each other, that is, the free energy is representative of the attraction between the substances involved. The free energy of attraction between water and hexane or octane obtained in this way at 25°C is about -40 erg/cm^2 of contact area, the free energy of attraction of the hydrocarbons for themselves at the same temperature is also about -40 erg/cm^2, but the free energy of attraction of water for itself is -144 erg/cm^2. It is clearly the latter alone that leads to a thermodynamic preference for elimination of hydrocarbon-water contacts; the attraction of hydrocarbon for itself is essentially the same as its attraction for water.

It should be noted that the correct interpretation of the hydrophobic effect, assigning the predominant role to the properties of water per se, has been given in several places: by Hartley (1936) in an elegant monograph on micelle formation in aqueous solution, by Frank and Evans (1945) in their interpretation of the thermodynamic properties of aqueous solutions of all kinds of hydrophobic or partly hydrophobic substances, and by Kauzmann (1959) in his analysis of the forces stabilizing the native structure of proteins.

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TWO

THE SOLUBILITY OF HYDROCARBONS IN WATER

The simplest hydrophobic substances include inert gases, hydrocarbons, and some other nonpolar organic substances. The amphiphilic substances that form micelles are nearly all substituted hydrocarbons, and the biological lipids that play the major role in the formation of cell membranes all contain aliphatic hydrocarbon chains as their hydrophobic entity. Thus, among simple hydrophobic substances, hydrocarbons are closest to the main theme of this book. This chapter provides a measure of their hydrophobicity, in terms of the free energy of transfer of hydrocarbon molecules from water to a purely hydrocarbon solvent. For straight-chain aliphatic hydrocarbons, this free energy will be seen to be a strictly linear function of the number of CH_2 groups in the chain. More generally, it is at least approximately proportional to the surface area of the cavity created in the solvent by the hydrophobic solute, or to the number of water molecules forced to be in contact with hydrocarbon.

For reasons first given by Gurney (1953) and reiterated by Kauzmann (1959), free energies of transfer from one solvent to another should be expressed in *unitary* units, which simply means that the solute concentration in the equation for the chemical potential is expressed in mol fraction units. Thus, for a hydrocarbon dissolved in water

$$\mu_W = \mu_W^\circ + RT \ln X_W + RT \ln f_W \qquad (2\text{-}1)$$

where X_W is the concentration of solute in mol fraction units, f_W the activity coefficient at that concentration, and μ_W° the standard chemical potential on the unitary scale. The reference state is the state of infinite dilution *in water*, which means that all interactions of an isolated hydrocarbon molecule with the solvent water are included in the term μ_W°, and $RT \ln f_W$ represents only that part of the excess chemical potential that arises from interactions of solute molecules with each other. Because of the very low solubility of hydrocarbons in water, $RT \ln f_W$ can safely be set equal to zero at actual experimental concentrations.

The advantage of using mol fraction units for the solute concentration lies in the fact that $RT \ln X_W$ is the correct expression (in very dilute solutions) for the *cratic* part of the chemical potential, which is the purely statistical contribution to the chemical potential that arises from the entropy of mixing solvent and solute molecules. The standard potential μ_W° thus contains no contributions from this effect and represents *only* the internal free energy of the solute molecule and the free energy of its interaction with the solvent. If some other concentration units had been used, μ_W° would include the contribution made by the entropy of mixing (on the basis of ideal mixing) at unit concentration, for example, at 1 mol/liter if molar concentrations are used. There would be a similar factor in the standard chemical potential in a hydrocarbon solvent (μ_{HC}°), but since 1 liter of water and 1 liter of liquid hydrocarbon generally contain different numbers of mols of solvent, the two cratic contributions would differ. Thus $\mu_{HC}^\circ - \mu_W^\circ$ would not represent the true difference between the inherent properties of the solute in the two solvents. If mol fraction units are used, μ° represents only the internal free energy of the solute molecule and the free energy of interaction with solvent, and, since the internal free energy is the same in both solvents, $\mu_{HC}^\circ - \mu_W^\circ$ is exactly the quantity we want, the difference between the free energy of interactions with the solvent.

To relate μ_{HC}° to experimental data we use the analog of equation 2-1 for solution of a hydrocarbon in a hydrocarbon solvent

$$\mu_{HC} = \mu_{HC}^\circ + RT \ln X_{HC} + RT \ln f_{HC} \tag{2-2}$$

This equation can be applied to a dilute solution of one hydrocarbon in another. In principle a different value of μ_{HC}° would apply for each distinct solvent, but it is reasonable to assume that differences between similar solvents are small, that is, μ_{HC}° in, say *n*-hexane and *n*-decane, can be assumed to differ little from each other in comparison with the difference between μ_{HC}° and μ_W°. This means that equation 2-2 can be applied to a solution of a hydrocarbon in itself, that is, to the pure liquid hydrocarbon. In this case both $\ln X_{HC}$ and $\ln f$ would be zero and μ_{HC} would be equal to μ_{HC}°. Accurate thermodynamic data are available for solutions of smaller *n*-alkanes in *n*-hexadecane and *n*-octadecane (McGlashan and Williamson, 1961; Cruickshank et al., 1968; Hicks and Young, 1968), and they show that the difference between μ_{HC}° in the pure liquid and μ_{HC}° at infinite dilution in the longer hydrocarbon is only about 60 cal/mol (near room temperature) when the solute and solvent differ in chain length by 10 carbon atoms. The result is roughly proportional to chain length difference, that is, it amounts to 6 cal/mol per CH_2 group. This is less than the experimental error in the determination of the dependence of $\mu_{HC}^{\circ} - \mu_{W}^{\circ}$ on hydrocarbon chain length (see below).

ALIPHATIC HYDROCARBONS

Equations 2-1 and 2-2 may be applied to the extensive solubility measurements of liquid hydrocarbons in water at 25°C by McAuliffe (1966). Since $\mu_W = \mu_{HC}^{\circ}$ at saturation, $\mu_{HC}^{\circ} - \mu_{W}^{\circ} = RT \ln X_w$. Results for a homologous series of *n*-alkanes are shown in Fig. 2-1 and are seen to be a linear function of the length of the hydrocarbon chain. A least-squares treatment leads to the relation

$$\mu_{HC}^{\circ} - \mu_{W}^{\circ} = -2436 - 884n_{\mathrm{C}} \tag{2-3}$$

where n_C is the number of carbon atoms in the hydrocarbon chain and μ° is expressed in cal/mol. The variance in the constant term is ±55 cal/mol, while that in the slope is ±13 cal/mol. The equation may be rewritten in terms of the contributions of the two terminal methyl groups and the internal CH_2 groups

$$\mu_{HC}^{\circ} - \mu_{W}^{\circ} = -2102n_{\mathrm{CH_3}} - 884n_{\mathrm{CH_2}} \tag{2-4}$$

but this must be done with the reservation that the equation applies only to straight-chain hydrocarbons, and that the coefficients of $n_{\mathrm{CH_3}}$ and $n_{\mathrm{CH_2}}$ do not represent intrinsic measures of the hydrophobic effect for CH_3 and CH_2 groups on more complex hydrocarbons. Thus $\mu_{HC}^{\circ} - \mu_{W}^{\circ}$ for isobutane is -5850 cal/mol: if equation 2-4 were generally applicable, the three CH_3 groups alone would contribute -6300 cal/mol. Another example is provided by cyclopentane, for which $\mu_{HC}^{\circ} - \mu_{W}^{\circ}$ is -6000 cal/mol, compared to -4420 cal/mol as would be calculated from equation 2-4.

In fact, $\mu_{HC}^{\circ} - \mu_{W}^{\circ}$ is probably closely proportional to the number of water molecules that must be in contact with the dissolved hydrocarbon molecule in water solution. When straight-chain hydrocarbons are compared, the first member of the series is ethane, which would clearly have to make a much larger number of such contacts than the number of new contacts created by subsequent insertion of CH_2 groups between the methyl groups. On the other hand, replacement of a hydrogen atom by a methyl group, as in the formation of isobutane from propane, obviously

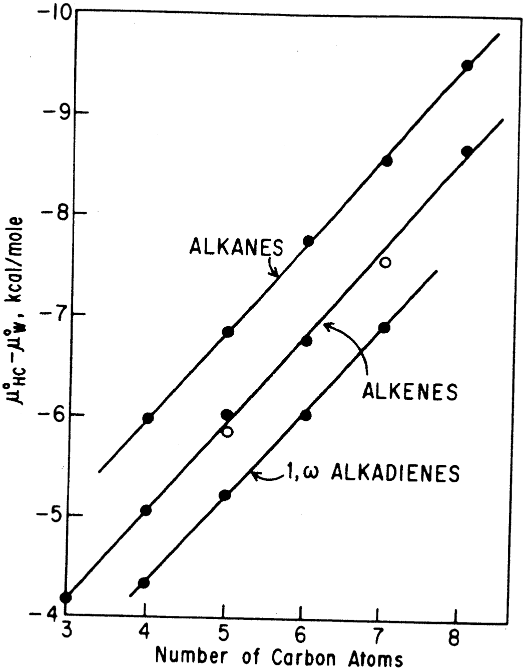

Fig. 2-1. Standard free energies for transfer of hydrocarbons from aqueous solution to pure liquid hydrocarbon at 25°C, based on solubility measurements of McAuliffe (1966). For the alkenes, filled circles represent 1-alkenes, and open circles represent 2-alkenes.

creates only a small increment in solvent contacts (much of the new area of contact would involve H_2O molecules already adjacent to the terminal CH_3 groups of propane). Experimental results for a variety of linear, branched, and cyclic hydrocarbons do in fact give an approximately linear plot against the hydrocarbon surface area or the number of water molecules in contact with it (Hermann, 1972; Reynolds et al., 1974). The slope is about 25 cal/mol per Å^2 of surface area. There is uncertainty in the absolute numerical value because of ambiguity in the definition of the interface between a solute molecule and the surrounding solvent and in the measurement of its area. The slope of 25 cal/mol per Å^2 was obtained when the area was calculated at a distance of 1.5 Å from the solute surface, this being the distance of closest approach of the centers of water molecules to the surface.

Linear relations of the form of equation 2-3 apply to homologous series of various kinds which are generated by increasing the length of a linear alkyl chain. For example, Fig. 2-1 shows free energies calculated from solubilities of liquid *n*-alkenes and *n*-dienes in water (McAuliffe, 1966). It is seen that the position of the double bond in the chain has a small effect on the results, points for 2-alkenes lying slightly below those for 1-alkenes. A least-squares treatment of the combined data leads to the line drawn in the figure, corresponding to the relation

$$\mu^\circ_{HC} - \mu^\circ_W = -1503 - 884n_C \qquad (2\text{-}5)$$

with a variance of ±120 in the constant term and ±22 in the slope. All the dienes investigated had double bonds in the terminal positions. The least-squares relation is

$$\mu^\circ_{HC} - \mu^\circ_W = -903 - 860n_C \qquad (2\text{-}6)$$

with probable error about the same as for equation 2-3. It is obvious that introduction of one or more double bonds has considerable influence on the absolute value of $\mu^\circ_{HC} - \mu^\circ_W$, but virtually none on the dependence on n_C. In fact, the constancy of the increment in $\mu^\circ_{HC} - \mu^\circ_W$ per added CH_2 group, as the chain length increases, or as branching points or double bonds are inserted, is quite remarkable. It must signify that interactions with solvents are of short range, and that interaction with water in particular cannot depend significantly on the formation of cooperative linkages of water molecules with special space requirements.

The effect of unsaturation on the constant terms of equations 2-5 and 2-6 (compared to equation 2-3) indicates that an unsaturated hydrocarbon is less hydrophobic than a saturated one. Introduction of one double bond is essentially equivalent to removing one CH_2 group from a fully saturated chain. Introduction of a second double bond has a somewhat smaller effect, but all the data refer to dienes with terminal double bonds, and it is possible that larger effects would be observed (as indicated by the results for alkenes) if the double bonds were internal.

In equations 2-5 and 2-6 the organic solvent to which μ°_{HC} refers (i.e., the pure liquid) is of course not a saturated hydrocarbon, and the results may therefore differ from those that would apply to solution in a saturated hydrocarbon. The increments per carbon atom indicate that any such difference is negligible insofar as CH_2 groups are concerned. The constant term might be affected more as a result of possible interaction between one double bond and another. The available evidence indicates that any effect from this source will be small. For example, μ° values for ethylene in *n*-hexane and in benzene differ by only 100 cal/mol.

AROMATIC HYDROCARBONS

Since we are primarily interested in these data in relation to biological membranes, the aromatic hydrocarbons are of peripheral importance only. Some results are tabulated in Table 2-1, and they show that aromatic hydrocarbons are considerably less hydrophobic than cyclic aliphatic hydrocarbons with the same number of carbon atoms. It must again be borne in mind that μ°_{HC} refers to the pure liquid, and that $\mu^\circ_{HC} - \mu^\circ_W$ for transfer of an aromatic solute from water to a liquid-saturated aliphatic hydrocarbon may differ from the figures given in this table. It is inconceivable, however, that this difference could amount to 2 kcal/mol, as can be seen from references provided by Rowlinson (1969). The major factor responsible for the difference between cyclohexane and benzene must arise from interactions with water, that is, the hydrophobicity is less for benzene because its π-electrons lead to stronger van der Waals attraction to water molecules.

The effect of adding a methyl group to an aromatic ring is only slightly different from the effect of adding a methyl group to a cyclic aliphatic hydrocarbon; the difference between toluene and benzene is 810 cal/mol, the difference between methyl cyclohexane and cyclohexane is 900 cal/mol. The effect of adding a methyl group to a normal alkane depends on how close it is to an already existing methyl group (see earlier discussion):

Table 2-1. $\mu^\circ_{HC} - \mu^\circ_W$ for Aromatic and Saturated Cyclic Hydrocarbons at 25°C[a]

	$\mu^\circ_{HC} - \mu^\circ_W$ (cal/mol)
Cyclopentane	−6000
Methyl cyclopentane	−6880
Cyclohexane	−6730
Methyl cyclohexane	−7630
Benzene	−4620
Toluene	−5430
Ethylbenzene	−6230
o-xylene	−6180
m-xylene	−6110
p-xylene	−6100

[a] Based on solubilities of the liquid hydrocarbons in water as determined by McAuliffe (1966), except the results for *m*- and *p*-xylene, which are based on measurements of Bohon and Claussen (1951).

the difference between 2-methyl butane and butane is 750 cal/mol, that between 2-methyl pentane and pentane is 710 cal/mol, but the difference between 2,4-dimethyl pentane and 2-methyl pentane is 880 cal/mol.

Gill and Wadsö (1976) have shown that a reasonably good correlation can be obtained, to include unsaturated and even aromatic hydrocarbons as well as linear and nonlinear saturated alkanes, if $\mu^\circ_{HC} - \mu^\circ_W$ is considered to be a linear function of the number of H atoms per molecule instead of the surface area. The theoretical justification for a relation of this kind is weak.

SOLUBILITY IN AQUEOUS SALT SOLUTIONS

It is well known that salts decrease the solubility of nonpolar substances in water, which means that μ° in aqueous salt solution is more positive than μ°_W. However, the effect is appreciable at high salt concentrations only. On the basis of results for ethane, propane, and butane (Morrison and Billett, 1952), the difference between μ° in a 1 molal salt solution and μ°_W at 25°C is approximately given by the relations

$$\begin{aligned} \Delta\mu^\circ &= -231 n_{\mathrm{CH_3}} - 38 n_{\mathrm{CH_2}} && \text{for NaCl} \\ \Delta\mu^\circ &= -181 n_{\mathrm{CH_3}} - 31 n_{\mathrm{CH_2}} && \text{for LiCl} \\ \Delta\mu^\circ &= -149 n_{\mathrm{CH_3}} + 4 n_{\mathrm{CH_2}} && \text{for KI} \end{aligned} \tag{2-7}$$

Differences between different salts are related to charge type and ionic radius.

The effect of salts on solubility is roughly proportional to salt concentration, and equations 2-7 thus show that $\Delta\mu^\circ$ is no larger than the uncertainty in $\mu^\circ_{HC} - \mu^\circ_W$ as given by equations 2-3 to 2-6 when the concentration of a uniunivalent salt is about 0.3 M or less. This means that we can assume the hydrophobic effect of *dilute* salt solutions to be essentially the same as that of pure water, and we shall therefore not make a distinction between them, that is, μ°_W will be used to refer to pure water or a dilute salt solution.

SOLUBILITY IN ALCOHOLS AND OTHER ORGANIC SOLVENTS

Nonpolar organic solvents such as CCl_4 are very close to hydrocarbons in their solvent properties. For example, $\mu^\circ_{\mathrm{CCl_4}} - \mu^\circ_W$ for ethane is the same, within experimental error, as $\mu^\circ_{HC} - \mu^\circ_W$. However, if an organic solvent contains polar molecules, it will have some structural organization, and may resist the intrusion of nonpolar solute molecules. No known solvent has this property to the same extent as water does, but glycerol and ethylene glycol, for example, repel nonpolar substances quite effectively (Sinanoglu and Abdulnur, 1965).

The aliphatic alcohols are relatively benign solvents for hydrocarbons, presumably because the local molecular organization segregates the hydrocarbon tails from the OH groups, so that no major reorganization is required to accommodate hydrocarbon molecules, at least at low concentrations. Nevertheless, some reorganization is required and μ°_{ROH} (subscript ROH refers to alcohol as the solvent medium) is distinctly more positive than μ°_{HC}. This can be seen from the results in Table 2-2, which show the difference between μ°_{ROH} and μ°_{HC} for propane and butane in several alcohols. It is seen that the difference is relatively small and decreases as the hydrocarbon portion of the alcohol increases in size. In the last two columns of this table values of $\mu^\circ_{\mathrm{ROH}} - \mu^\circ_W$ are compared with values of $\mu^\circ_{HC} - \mu^\circ_W$, and it is seen, with reference to equation 2-4 or 2-5, that the difference between these quantities lies more in the constant term of the equation than in the increment per carbon atom. The difference between $\mu^\circ_{ROH} - \mu^\circ_{\mathrm{W}}$ for butane and propane is 750, 800, and 830 cal/mol, respectively, in the three alcohols. The corresponding difference in $\mu^\circ_{HC} - \mu^\circ_W$ is 890 cal/mol.

Table 2-2 contains some rough data for glycerol and ethylene glycol, which show that a strong solvophobic effect is associated with these solvents, especially with glycerol, which is almost as antagonistic to butane as water is.

Table 2-2. Free Energy of Transfer from Alcohols to Water at 25°C[a]

Hydrocarbon	Solvent	$\mu^\circ_{\mathrm{ROH}} - \mu^\circ_{HC}$ (cal/mol)	$\mu^\circ_{\mathrm{ROH}} - \mu^\circ_W$ (cal/mol)	$\mu^\circ_{HC} - \mu^\circ_W$ (cal/mol)
Propane	MeOH	1460	−3620	−5080
	EtOH	1100	−3980	
	isoPrOH	880	−4200	
Butane	MeOH	1600	−4370	−5970
	EtOH	1190	−4780	
	isoPrOH	940	−5030	
	Glycol[a]	3700	−2300	
	Glycerol[a]	4800	−1200	

[a] The figures for butane in glycol and glycerol are based on data of uncertain reliability, taken from the Landolt-Börnstein Tabellen (1960). All other results are based on the work of Kretschmer and Wiebe (1952), and represent accurate data, extrapolated to infinite dilution of the hydrocarbon in the alcohol solution.

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THREE

SOLUBILITY OF AMPHIPHILES IN WATER AND ORGANIC SOLVENTS

Ionic or polar derivatives of hydrocarbons were called *amphipathic* by Hartley (1936) because they contain one part that has "sympathy" and another that has "antipathy" for water. We have preferred in this book to use the alternative and more euphonious term *amphiphilic*. To avoid repetition of the clumsy phrase "amphiphilic molecule or ion," we shall use the noun *amphiphile* as designating either an ionic or a molecular species. The polar or ionic group of an amphiphile is generally called the *head*, and the hydrocarbon chain is called the *tail*. Because solvent-solute interactions are of quite short range, the solvent-dependent part of the chemical potential of such molecules may be thought of as the sum of nearly independent contributions from the tail and the head. As the length of the tail is increased, its contribution becomes dominant, and the amphiphile becomes progressively less soluble in aqueous solutions.

At one position on an amphiphilic ion or molecule, the point of attachment of the tail to the head, the hydrophilic and hydrophobic properties are in conflict. Since the ordering of water molecules by the polar head is the result of strong attractive forces, the influence of the hydrophilic head group is likely to predominate. Thus one CH_2 group of the hydrophobic tail (proximal to the head group) may be expected to make little or no contribution to the hydrophobic effect, but there is no evidence to suggest that the influence of the head group can make itself felt at any greater distance from the point of attachment.

The equilibrium distribution of fatty acids between *n*-heptane and aqueous buffer solutions, ionic strength 0.1 to 0.15, has been studied by Goodman (1958) and by Smith and Tanford (1973). As noted earlier, the presence of salts at relatively low concentrations should not significantly affect hydrocarbon-water interactions, and the results can be considered equivalent to those that would have been obtained if the aqueous phase had been pure water. Using equation 2-1 for the chemical potential of the solute in the aqueous solution and equation 2-2 for the solution in heptane, we have at equilibrium

$$\mu^{\circ}_{HC} - \mu^{\circ}_{W} = RT \ln X_W/X_{HC} + RT \ln f_w/f_{HC} \tag{3-1}$$

and the desired free energy difference can be obtained from the distribution ratio X_W/W_{HC}.

There are experimental complications, in that X_W/X_{HC} is very small for acids with long alkyl chains. To have a measurable concentration of solute in the aqueous phase while keeping X_{HC} reasonably small, it is necessary to use a high pH, so that most of the solute in the aqueous phase will be in the form of the anion, $RCOO^-$, the actual value of X_W being calculated from the measured concentration by use of the acid dissociation constant. Furthermore, there is a strong tendency for association in both solvents: in the organic phase, dimers of RCOOH are formed with hydrogen bonds between the head groups, and in the aqueous phase there is strong association between RCOOH and $RCOO^-$. As a result, considerable care has to be exercised to ascertain that experimental data are extended to conditions where the activity coefficient term in equation 3-1 can be neglected.

The results obtained are shown in Fig. 3-1. The earlier data of Goodman (1958) appeared to indicate that a linear relation between $\mu^{\circ}_{HC} - \mu^{\circ}_{W}$ and alkyl chain length extends only as far as palmitic acid, and this led Mukerjee (1967) to suggest that there might be a limit to the hydrophobicity of a hydrocarbon chain, possibly indicating that chains with more than 16 carbon atoms fold upon themselves in aqueous solution, so that no appreciable further increase in the hydrocarbon-water interface occurs. The recent data of Smith and Tanford (1973) have shown, however, that Goodman's results for palmitic and stearic acids were obtained under conditions where association between RCOOH and $RCOO^-$ in the aqueous phase still affects the distribution. When this difficulty is avoided the linear relation between $\mu^{\circ}_{HC} - \mu^{\circ}_{W}$ persists at least up to behenic acid (22 carbon atoms). Least-squares analysis of the data, ignoring Goodman's results for the higher acids, leads to the relation (in cal/mol)

$$\mu^{\circ}_{HC} - \mu^{\circ}_{W} = 4260 - 825 n_C \tag{3-2}$$

The constant term on the right-hand side of equation 3-2 is positive,

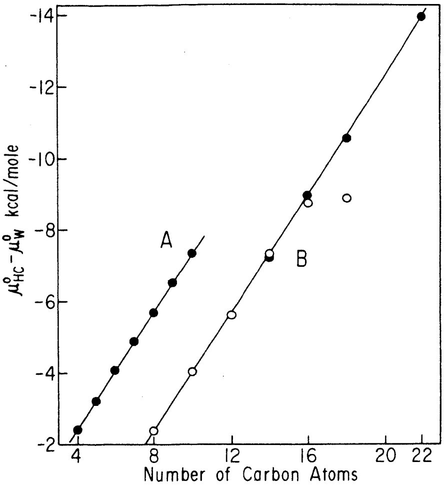

Fig. 3-1. Curve A represents free energies for transfer of pure aliphatic alcohols from aqueous solution to pure liquid alcohol at 25°C, based on data of Kinoshita et al. (1958). Curve B represents free energies of transfer of undissociated fatty acids from a dilute aqueous buffer solution to liquid *n*-heptane at 23° to 25°C. Open circles represent data of Goodman (1958), and filled circles represent data of Smith and Tanford (1973).

reflecting the high polarity and consequent preference for water of the COOH group. If we assume that the terminal methyl group of the alkyl chain makes the same contribution to $\mu^\circ_{HC} - \mu^\circ_W$ as in a pure hydrocarbon (− 2100 cal/mol), the formal contribution from the COOH group becomes +4700 cal/mol. If we wish to ascribe part of this to the likely ineffectiveness of one CH_2 group, assuming that it makes no contribution to $\mu^\circ_{HC} - \mu^\circ_W$ because the water around it (in the aqueous phase) may be associated with the amphiphile head group, then the calculated contribution due purely to hydration of the COOH group in water would become +3900 cal/mol.

The dependence of $\mu^\circ_{HC} - \mu^\circ_W$ on n_C reflects the hydrophobic interactions of CH_2 groups inserted in the chain as the chain length is increased from 8 to 22 carbon atoms. The value obtained, 825 cal/mol, is somewhat smaller than the corresponding figures from equations 2-3, 2-5, and 2-6, but the difference is not much greater than the experimental uncertainty.

In addition to the results shown in Fig. 3-1, Goodman (1958) obtained distribution data for oleic and linoleic acids, which are C_{18} acids with one and two double bonds, respectively. The results indicate that the introduction of double bonds decreases the magnitude of hydrophobic interactions, as was observed with pure hydrocarbons. A quantitative comparison cannot be made because of the previously mentioned error in Goodman's results for stearic acid.

Figure 3-1 also shows data based on the solubilities of aliphatic alcohols in water (Kinoshita et al., 1958). The saturating phase was the pure liquid alcohol, and the chemical potential in that phase is represented by μ°_{ROH}. The results may be expressed as

$$\mu^\circ_{\mathrm{ROH}} - \mu^\circ_W = 833 - 821 n_{\mathrm{C}} \tag{3-3}$$

with a variance of only ±3 cal/mol in the slope, and ±25 cal/mol in the constant term. It is seen that the increment per CH_2 group is the same as that in equation 3-2, even though the organic solvent in this case is not a pure hydrocarbon. This agreement may be attributed to the probable hydrogen bonding between OH groups in a liquid alcohol, which would leave the distal portions of hydrocarbon tails essentially in a hydrocarbon environment. (See discussion of solubilities of hydrocarbons in alcohols in Chapter 2.) The constant term in equation 3-3 is very much smaller than that for carboxylic acids, given in equation 3-2, which is of course due to the fact that the OH group in this case is hydrogen bonded in the organic solvent as well as in water. Data (of somewhat poorer quality) for the distribution of aliphatic alcohols between *n*-heptane and water (Gilbert et al., 1975) fall close to line *B* of Fig. 3-1 and indicate that the formal contribution of an OH group to $\mu^\circ_{HC} - \mu^\circ_W$ is similar to that of a COOH group, between +4 and +5 kcal/mol.

Davis et al. (1972, 1974) have analyzed distribution experiments and related data for a very large number of organic compounds, most of them with smaller hydrophobic moieties than in the molecules used for the data of Fig. 3-1. They obtained −850 ± 40 cal/mol for the contribution of CH_2 groups, about +5 kcal/mol for NH_2, OH, and other polar groups, and about +3.5 kcal/mol for an ether oxygen atom. Harris et al. (1973) have made distribution measurements for a series of amphiphiles with the same head group and with a variety of linear and nonlinear hydrocarbon tails. They demonstrated that the hydrophobic contribution to the free energy is a linear function of the surface area of contact with water, in agreement with the similar conclusion reached for pure hydrocarbons in the preceding chapter.

These results support the idea expressed at the beginning of this chapter, that the contributions of the hydrophilic and hydrophobic portions of an amphiphile to the free energy of interaction with solvent should be nearly independent. One might have expected the increment per carbon atom in equation 3-2 to be a few percent larger, that is, the same as in equations 2-3, 2-5, and 2-6. However, it must be recognized that we are dealing with isolated experimental studies and that the absolute accuracy of the measurements may not be as good as the self-consistency expressed by the close adherence to the linear relations.

THE ACTIVITY COEFFICIENT

The measurements on which equations 3-2 and 3-3 and the similar equations of Chapter 2 are based were made at very low values of X_W and extrapolated to infinite dilution, so that $\ln f_W$ in equation 2-1 could be set equal to zero. In the case of the carboxylic acids, considerable experimental difficulty was encountered in achieving a sufficiently low solute concentration to permit an extrapolation to where results become independent of concentration (Smith and Tanford, 1973). This and many other data indicate that significant interactions between amphiphiles in aqueous solution occur already at very low solute concentrations. Such deviations can be expressed in terms of an excess chemical potential or a nonzero value for $\ln f_W$, but this can only be an interim solution to the problem. One aims where possible to replace $\ln f_W$ by a term in the equation that reflects the events responsible for thermodynamic nonideality on a molecular basis. It is often possible to account for nonideality in large part by the formation of new chemical species, and to replace $\ln f_W$ by expressions involving the equilibrium constants for their formation. Scatchard (1976) has used the term *species ideal* to describe solutions for which this is true.

The major new species formed in aqueous solutions of amphiphiles are micellar aggregates, and, by making allowance for their formation as distinct species, all or the major part of nonideality in these solutions is readily accounted for. Micelles will be discussed in detail in later chapters of this book; the reason for mentioning the subject here is that there is evidence (at least for amphiphiles with small hydrophobic moieties) that a different kind of interaction between solute molecules can occur at solute concentrations below those required for micelle formation. The most compelling evidence (Nakanishi, 1960; Franks and Smith, 1968; Bruun and Hvidt, 1977; Hvidt et al., 1978) comes from the measurement of volume changes in solutions of small amphiphile molecules which do not form micelles of sufficient size to be detectable by the experimental methods normally used to detect large solute particles. Nevertheless, they undoubtedly form small micellelike clusters at high solute concentration, and this should be accompanied by an increase in partial molar volume, because micelles are aggregates in which the hydrophobic moieties of the amphiphile self-associate so as to minimize contact with water, and elimination of such hydrophobic contacts always leads to a volume increase, as is discussed in the following chapter (p. 26). The partial molar volume of the solute does in fact increase, and at high concentration it approaches the molar volume in pure bulk liquid.

As the typical data of Fig. 3-2 show, however, there is an initial marked volume decrease at low solute concentrations. Its dependence on con-

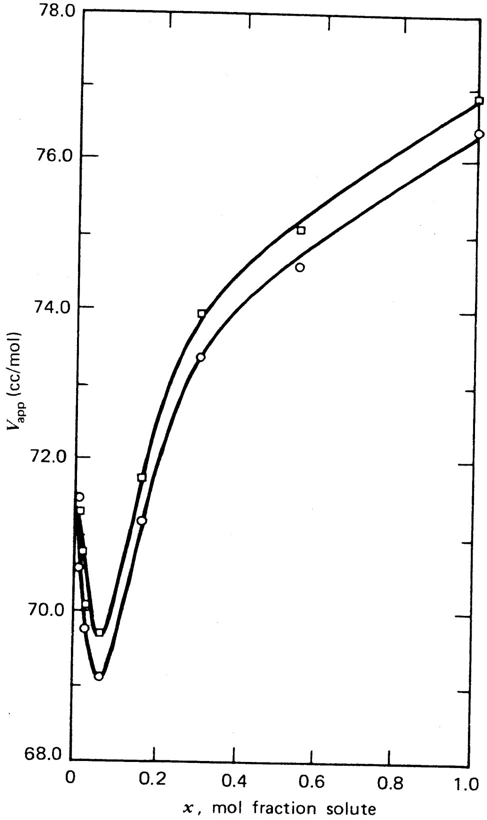

Fig. 3-2. The apparent molar volume of 2-propanol in aqueous solution, as a function of the solute mol fraction, at 20° (lower curve) and 25°C (Bruun and Hvidt, 1977). The apparent molar volume is an integral volume that becomes equal to the thermodynamic partial molar volume at $x = 0$ and $x = 1$.

centration indicates that it might be caused by association between solute molecules, and it has been suggested that dimers are the dominant associated form (Franks, 1975). The hydrocarbon moieties of the solute have to be involved in some way because the anomalous volume change is not observed for solutes such as urea and formamide, which lack hydrocarbon moieties, and in a homologous series the anomaly becomes more prominent as the size of the hydrocarbon moiety is increased. However, the sign of the volume change clearly shows that simple elimination of hydrophobic contacts with water cannot be the driving force for the association. Thus there is clearly an unexplained source of nonideality in these solutions, which, in the equation for the chemical potential, must at present be taken into account in terms of the activity coefficient.

Whether the type of interaction reflected in these volume measurements continues to be important for amphiphiles with long alkyl chains is uncertain, but there is evidence to suggest that it does not. Micelles are formed at progressively lower solute concentrations as the size of the hydrocarbon moiety of an amphiphile is increased (Chapter 7). Thus a monomer concentration sufficient for the alternate type of interaction may therefore not be possible. Density measurements for some micelle-forming amphiphiles (Tanford et al., 1974; Fig. 1) suggest that there are no anomalous volume changes in the limited range of concentration below that at which micelles are formed, and evidence for premicellar association of Na dodecyl sulfate based on conductance measurements (Mukerjee et al., 1958; Mukerjee, 1967) has been disputed (Parfitt and Smith, 1962).

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FOUR

THE EFFECT OF TEMPERATURE

ANOMALOUS ENTROPY AND HEAT CAPACITY

The effect of temperature on the free energy of any process allows one to determine the separate contributions of energy (or enthalpy) and of entropy to the free energy. This is often very helpful in arriving at a qualitative molecular mechanism for the process, and this is especially true for the hydrophobic effect. The classical mechanistic representation of the hydrophobic effect by Frank and Evans (1945) is based primarily on such considerations, and more sophisticated approaches to the problem have been able to add little to the original picture, for reasons to be discussed in Chapter 5. In this chapter we summarize the essential points made by Frank and Evans (insofar as they refer to hydrocarbon chains), basing the discussion, however, on more recent experimental data.

The relation between $\mu^\circ_{HC} - \mu^\circ_W$ and the corresponding partial molal enthalpies and entropies is

$$\mu^\circ_{HC} - \mu^\circ_W = \bar{H}^\circ_{HC} - \bar{H}^\circ_W - T(\bar{S}^\circ_{HC} - \bar{S}^\circ_W) \tag{4-1}$$

The separate contributions can be evaluated if the free energy difference is determined as a function of temperature, since

$$\frac{d[(\mu^\circ_{HC} - \mu^\circ_W)/T]}{d(1/T)} = \bar{H}^\circ_{HC} - \bar{H}^\circ_W \tag{4-2}$$

Alternatively, the enthalpy difference can be measured directly by calorimetry. In either case, the entropy difference is obtained by difference from equation 4-1. Since we shall use the data as a basis for discussing the state of the hydrocarbon in the aqueous medium, it will be convenient to consider the thermodynamic functions as they apply to the transfer process in the direction opposite to that considered thus far, that is, as $\mu^\circ_W - \mu^\circ_{HC}$, $\bar{H}^\circ_W - \bar{H}^\circ_{HC}$, and so on.

The effect of temperature on the aqueous solubility of hydrocarbons, or on the distribution of hydrocarbon between water and other solvents, always leads to curved plots, as illustrated by Fig. 4-1. The results are quite unexpected in two ways. (1) The solubility of the smaller aliphatic hydrocarbons near room temperature decreases as the temperature rises, and $\bar{H}^\circ_W - \bar{H}^\circ_{HC}$ is therefore negative. (For aromatic hydrocarbons the enthalpy change is slightly positive at 25°C.) This means that the transfer of the hydrocarbon to water is *energetically favored* (or, for aromatic hydrocarbons, energetically neutral), and that the positive value of $\mu^\circ_W - \mu^\circ_{\text{org}}$ results from a large negative entropy change in the process. Since the data are in unitary units, this must be an inherent entropy change involving an ordering process in the solution, and cannot be associated with dispersal of the solute per se. (2) The data display remarkable curvature, which of course persists when they are plotted against $1/T$ in the form of a van't Hoff plot. This means that the enthalpy change is itself strongly temperature dependent, and this in turn tells us that a large change in heat capacity accompanies the process, since

$$\frac{d(\bar{H}^\circ_W - \bar{H}^\circ_{\text{org}})}{dT} = (\bar{C}^\circ_p)_W - (\bar{C}^\circ_p)_{\text{org}} \tag{4-3}$$

When the temperature dependence of solubility or distribution measurements leads to curved plots as in Fig. 4-1, enthalpy and heat capacity data derived from the temperature dependence per se are subject to error, and direct calorimetric measurements are required to obtain accurate results. Accurate calorimetric measurements have been made by Gill et al. (1976), and they are summarized in Table 4-1. Entropy changes were calculated by use of equation 4-1. The table also contains some data (in parentheses) based on curve fitting of van't Hoff plots.

The enthalpy, entropy, and heat capacity values of amphiphilic molecules in water display similar features to those characteristic of pure hydrocarbons, as has been recognized for a long time. The anomalous heat capacities in particular are easily recognized from direct measurements, and attention to them was first drawn by Edsall (1935). Table 4-2 provides experimental data for some aliphatic alcohols. The enthalpy and heat capacity data were obtained by direct calorimetric measurement. The corresponding free energies of transfer are given by equation 3-3, and they have been used to obtain entropies of transfer by use of equation 4-1.

The negative enthalpies and entropies of Tables 4-1 and 4-2, and the very large anomalous heat capacities, provide considerable insight into

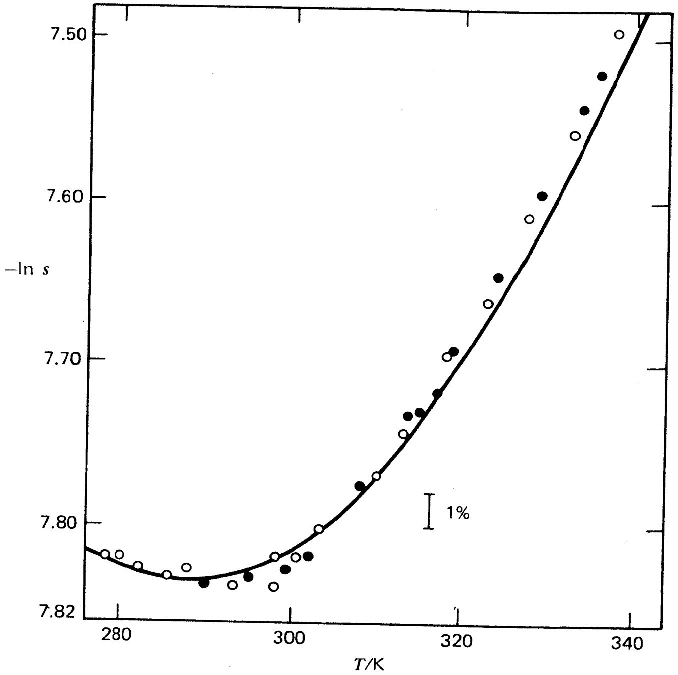

Fig. 4-1. The effect of temperature on the solubility of benzene in water, from Gill et al. (1976). Experimental data are from Arnold et al. (1958) and Franks et al. (1963), and the line is based on the thermodynamic paramaters for benzene in Table 4-1, assuming that the heat capacity change is independent of temperature. The small deviation of the experimental points from the curve at high temperature might reflect a slight temperature dependence of the heat capacity change. Reproduced with permission from the *Journal of Chemical Thermodynamics*. Copyright by Academic Press Inc. (London) Ltd.

the origin of the hydrophobic effect, which will be briefly discussed. It is apparent that the heat capacities for transfer to water are much larger than the heat capacities of the solute molecules themselves, as seen from the values for gaseous ethane and for other hydrocarbons in the liquid state, listed in the last column of Table 4-1. There is no possibility that such large increases in $\bar{C}_p$ can result from the effect of temperature on the internal motions of the solute molecules. They must be associated with changes in the state of water molecules brought about by the presence of the dissolved hydrocarbon.

Table 4-1. Thermodynamic Parameters for Transfer of Hydrocarbons from Organic Solvents to Water at 25°C[a]

	From CCl_4 or C_6H_6 to Water				
	$\mu^\circ_W - \mu^\circ_{org}$ (cal/mol)	$\bar{H}^\circ_W - \bar{H}^\circ_{org}$ (cal/mol)	$\bar{S}^\circ_W - \bar{S}^\circ_{org}$ (cal/deg mol)	$(\bar{C}^\circ_p)_W - (\bar{C}^\circ_p)_{org}$ (cal/deg mol)	$(\bar{C}^\circ_p)_{gas}$
C_2H_6[b]	3800	(−1800)	(−19)	(59)	12.6
C_2H_6[c]	3600	(−2200)	(−20)	(59)	12.6

	From Pure Liquid Hydrocarbon to Water				
	$\mu^\circ_W - \mu^\circ_{HC}$ (cal/mol)	$\bar{H}^\circ_W - \bar{H}^\circ_{HC}$ (cal/mol)	$\bar{S}^\circ_W - \bar{S}^\circ_{HC}$ (cal/deg mol)	$(\bar{C}^\circ_p)_W - (\bar{C}^\circ_p)_{HC}$ (cal/deg mol)	$(\bar{C}^\circ_p)_{HC}$
C_2H_6[d]	3900	(−2500)	(−21)	—	—
C_3H_8[d]	4900	(−1700)	(−22)	—	—
C_4H_{10}[d]	5900	(− 800)	(−23)	(65)	34
C_5H_{12}	6860	− 500	−25	96	41
C_6H_{14}	7740	0	−26	105	47
C_6H_6	4600	+ 500	−14	54	32
$C_6H_5CH_3$	5400	+ 410	−17	63	37
$C_6H_5C_2H_5$	6200	+ 480	−19	76	44
$C_6H_5C_3H_8$	6900	+ 550	−21	94	—

[a] Based on calorimetric data of Gill et al. (1976), except for results shown in parentheses.

[b] From CCl_4. Solubilities in the organic solvent from Hariutu, as quoted in the Landolt-Börnstein Tabellen (1962); solubilities in water from Morrison (1952). The subscript "org" is used in the headings because CCl_4 is not a hydrocarbon.

[c] From benzene. Source of data same as for CCl_4.

[d] Based on solubilities of the gaseous hydrocarbons in water (Morrison, 1952; Claussen and Polglase, 1952; Kresheck et al., 1965) together with extrapolated parameters for the vaporization of the corresponding liquids (American Petroleum Institute, 1953).

INTERPRETATION IN TERMS OF CHANGES IN WATER STRUCTURE

Water is a highly structured liquid, with hydrogen bonds linking the individual molecules to each other. The precise arrangement about each molecule is not known (see Chapter 5), but the tetrahedral symmetry of the oxygen bond orbitals and the tetrahedral structure of ice suggest a locally tetrahedral arrangement of molecules in the liquid state also. Any arrangement of this type would have to be disrupted by *any solute* dis-

Table 4-2. Thermodynamic Parameters for Transfer of Aliphatic Alcohols from the Pure Liquid to Water at 25°C[a]

	$\mu^\circ_W - \mu^\circ_{\text{ROH}}$ (cal/mol)	$\bar{H}^\circ_W - \bar{H}^\circ_{\text{ROH}}$ (cal/mol)	$\bar{S}^\circ_W - \bar{S}^\circ_{\text{ROH}}$ (cal/deg mol)	$(\bar{C}^\circ_p)_W - (\bar{C}^\circ_p)_{\text{ROH}}$ (cal/deg mol)
Ethanol	760	−2430	−10.7	39
n-propanol	1580	−2420	−13.4	56
n-butanol	2400	−2250	−15.6	72
n-pentanol	3222	−1870	−17.1	84

[a] Enthalpies and heat capacities were determined calorimetrically by Arnett et al. (1969). Values of $\mu^\circ_W - \mu^\circ_{\text{ROH}}$ were calculated by equation 3-3, and it should be noted that the direct data on which that equation is based extend only from butanol to decanol.

solved in water. Some hydrogen bonds would have to be broken: if the solute is polar, new hydrogen bonds between water and solute would be formed, but if the solute is nonpolar, such as a hydrocarbon, and unable to form hydrogen bonds, the net result expected is an increase in the enthalpy of the solution and, thereby, a positive value for $\mu^\circ_W - \mu^\circ_{\text{org}}$. The intrinsic difference between water and alcohols in this regard is worth noting. In a liquid alcohol the tendency for O—H···O hydrogen bond formation is likely to be as strong as in water, but such hydrogen bonds cannot comprise an isotropic network filling the entire solvent space. Hydrogen-bonded regions must alternate with regions composed of alkyl side chains, and a nonpolar solute can be dissolved in such a region without major disruption of solvent organization and, certainly, without disruption or appreciable distortion of hydrogen bonds.

It is evident from the results in Tables 4-1 and 4-2, and similar data for other nonpolar and amphiphilic molecules, that the intuitive explanation for the hydrophobic effect given in the previous paragraph is not adequate. The rupture of hydrogen bonds requires energy and, if the preceding explanation were correct, the major contribution to the free energy of transfer to water would be a positive enthalpy term. Instead, as we have seen, transfer to water may actually be slightly favored energetically, the positive value of $\mu^\circ_W - \mu^\circ_{\text{org}}$ resulting from a negative entropy change for the process. There is only one possible explanation, as was pointed out in a classic paper by Frank and Evans (1945): the water molecules at the surface of the cavity created by a nonpolar solute must be capable of rearranging themselves in order to regenerate the broken hydrogen bonds (in fact, where the enthalpy change is negative they may be slightly stronger than before), but in doing so they create a higher

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degree of local order than exists in pure liquid water, thereby producing a decrease in entropy.

The change in the sign of ΔH for aliphatic hydrocarbons as the molecular size increases (and the concomitant smaller change in ΔS) suggests that the local ordering of water molecules at a hydrocarbon–water interface is not unique, but that different arrangements become possible as the interfacial area increases. The linear dependence of $\mu_W^\circ - \mu_{HC}^\circ$ on hydrocarbon chain length or surface area may thus to some extent be fortuitous. The different arrangements must differ in enthalpy and entropy in a mutually compensating way, so that the hydrophobic free energy is not significantly affected.

The anomalous heat capacities are consistent with the existence of such multiple arrangements. If two such arrangements have nearly the same free energy, they must be about equally probable, that is, there would be a local equilibrium between two states of the solvent, one with a higher enthalpy *and* a higher entropy than the other. An increase in temperature would shift the equilibrium toward this higher enthalpy state, and lead to a higher enthalpy for the solution as a whole. Experimentally, this enthalpy increase would manifest itself as an anomalous contribution to the partial molal heat capacity $\bar{C}_p^\circ$ of the solute in the aqueous solution. One possibility is that in the "high enthalpy" state the cavity containing the solute is lined by broken hydrogen bonds (as in the intuitive model that these data have forced us to discard), but it is equally likely that the hydrogen bonds are weaker and less rigidly ordered, without any change in their number. The main conclusion is that the organization of water at a hydrocarbon-water interface is a makeshift arrangement, subject to alteration as a result of temperature changes as well as changes in hydrocarbon chain length.

PARTIAL MOLAR VOLUME

The ordering of water molecules around a hydrophobic solute, as reflected in the negative values for $\bar{S}_W^\circ - \bar{S}_{HC}^\circ$, is also accompanied by a decrease in volume (Friedman and Scheraga, 1965). For example, the partial molar volume of *n*-pentanol at infinite dilution in water at 20°C is 101.9 cc/mol, compared with 108.2 cc/mol for the molar volume of the pure liquid.

SOLUTIONS OF IONS IN WATER

As was noted above, *any solute* must disrupt the local ordering of solvent molecules in liquid water. It is therefore instructive, as a further aid to visualizing the structural implications of thermodynamic data for solutions of hydrocarbons in water, to compare these data with those for other solutes. Solutions of ions are particularly interesting in this regard, because they are also frequently characterized by negative entropy and volume contributions, indicative of the ordering of water molecules around the ions by ion-dipole forces. An example is provided by the reaction $H_2O + CH_3COOH \rightarrow H_3O^+ + CH_3COO^-$. The enthalpy change in this reaction is close to zero, because there is essentially no net change in the number or strength of hydrogen bonds. The value of ΔS° for the reaction at 25°C (in unitary units) is -30 cal/mol deg and the volume change is -17 cc/mol. As before, the only conceivable origin for this lies in the organization of solvent molecules. In this case, however, ΔC_p° for the reaction is also negative (-34 cal/deg mol), pointing to the intrinsic difference between the modes of organization induced by nonpolar and polar solutes. The water molecules near a nonpolar solute are hydrogen-bonded to each other in an intrinsically labile state (as indicated above), *more* labile than the ordered arrangement of water molecules in pure water. The water molecules surrounding an ion are firmly hydrogen-bonded, and *less* labile than they would be in pure water.

The picture is somewhat complicated by the fact that some metal ions have an even more negative partial molal heat capacity than can be accounted for in this way, but such ions also have an anomalous *positive* component to the partial molal entropy. Frank and Evans (1945) have interpreted this phenomenon as indicating the existence of a layer of disordered water molecules at the interface between a hydrated ion and the surrounding solvent.

THE SURFACE FREE ENERGY OF WATER

Water in contact with air tends to minimize its surface area by forming drops, that is, the transfer of a water molecule from bulk water to the surface is accompanied by an increase in free energy. Moreover, the surface formed has considerable two-dimensional strength: hydrogen bonds parallel to the surface undoubtedly form a stronger network than exist in any plane in bulk water. Intuitively this would seem to be a process resembling that which occurs at the interface between water and a hydrophobic solute. However, caution must be exercised in drawing a parallel between the two processes, for the enthalpy and entropy changes accompanying formation of a water-air interface are both positive, that is, although the hydrogen bonds in the plane of the surface of a drop of water are very strong, the total energy of a water molecule in the surface is greater, not less than the energy per molecule within the liquid.

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FIVE

THE STRUCTURE OF WATER[1]

We have seen that a qualitative picture of the structural changes that occur in water when a hydrophobic solute is added can be deduced from thermodynamic data alone. We should like of course to have a more definite, quantitative picture of the molecular rearrangements that take place, but to do so we must first have definite knowledge of the structure of pure liquid water. This chapter will present a very brief summary of present knowledge of the structure of water, and will show that our information is not complete enough to warrant an attempt to formulate a precise structural model for aqueous solutions.

ORDINARY ICE AND OTHER CRYSTALLINE FORMS

The most powerful tools for elucidation of exact structural information are X-ray and neutron diffraction of crystalline substances, and these methods have of course been applied to determine the structures of ordinary ice and of the other, denser crystal forms of water that are stabilized by high pressure. They have also been used to determine the structures of crystalline complexes of water with small nonpolar molecules (such as $Cl_2 \cdot 8\ H_2O$ and $CH_4 \cdot 5\frac{3}{4}\ H_2O$) that are formed when water is crystallized in the presence of these substances. The latter are of special interest to us since they allow us to see some possible arrangements of water molecules in the presence of hydrophobic molecules. The struc-

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[1] Much of this chapter is based on the book on the structure and properties of pure water by Eisenberg and Kauzmann (1969).

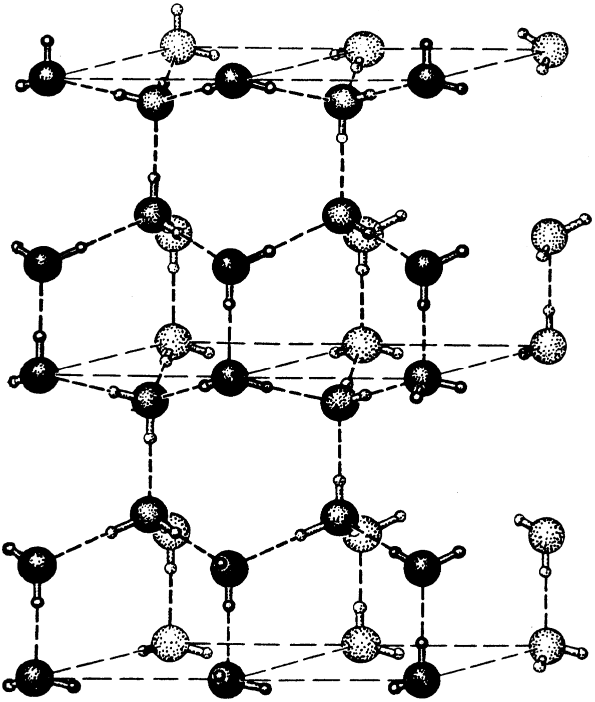

Fig. 5-1. The structure of ordinary ice. Large spheres represent oxygen atoms, and small spheres represent hydrogen atoms. The orientation of water molecules is arbitrary: alternative arrangements can arise from rotation of water molecules. Reprinted from Linus Pauling: *The Nature of the Chemical Bond.* Copyright 1939 and 1940 by Cornell University. Third edition © 1960 by Cornell University. Used by permission of Cornell University Press.

tures of ordinary ice and of one of the gas hydrates are shown in Figs. 5-1 and 5-2.

The hydrides of the smaller nonmetallic elements are gases at room temperature. H_2O is the sole exception, and its existence in condensed phases is due to the strength of O—H···O hydrogen bonds, and to the fact that each water molecule can form four such bonds, two in which it acts as a hydrogen donor and two in which it acts as a receptor. As a consequence of this arrangement, the crystal structure adopted by ordinary ice (Fig. 5-1) is a tetrahedral one, with a distance of about 2.76 Å between neighboring oxygen atoms. This arrangement is a relatively open one. The nearest nonhydrogen-bonded neighbors are 4.5 Å apart, and

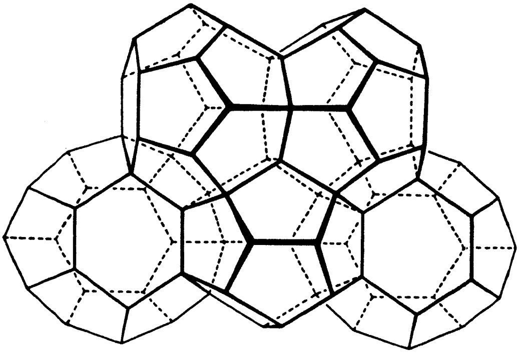

Fig. 5-2. A portion of the hydrogen bond framework of crystalline chlorine hydrate: each line represents an O—H· · ·O bond. Molecules of Cl_2 are contained in the larger (tetrakaidecahedral) cavities, but not in the dodecahedral ones. Reprinted from Linus Pauling: *The Nature of the Chemical Bond.* Copyright 1939 and 1940 by Cornell University. Third edition © 1960 by Cornell University. Used by permission of Cornell University Press.

much unfilled space is left within the crystal. This feature accounts for the low density of ice, one of relatively few crystalline materials that becomes more dense when it melts.[2] It should be noted that the H_2O molecule remains intact in the crystal: the H atoms do not move to positions halfway between adjacent O atoms, but remain about 1.00 Å from the O atom to which they "belong" and 1.76 Å from the O atom to which they form a hydrogen bond. These specifications still permit a vast number of possible orientational arrangements (which may be altered by cooperative rotations of the water molecules), and the crystals have been found to be disordered with respect to this feature, as was first suggested by Pauling (1935) on the basis of the residual entropy of ice at 0°K.

The unfilled space within normal ice crystals allows ice to respond to increased pressure by formation of higher density structures. Eight stable polymorphic-ordered forms are known, ranging in density up to 1.66 g/cc (Kamb, 1968). The water molecules in these denser crystals retain the basic hydrogen-bonded structure, with each water molecule hydrogen bonded to its four nearest neighbors. However, some of the hydrogen bonds are distorted (O—H· · ·O is no longer linear), and nonbonded water molecules are thereby permitted to approach much closer to each other than in ordinary ice: in ice II the closest nonhydrogen-bonded neighbors are 3.24 Å apart, in the densest polymorphs (ice VII and ice VIII) the distance between nonbonded neighbors and hydrogen-bonded neighbors becomes equal at 2.86 Å.

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[2] This property is shared by other substances with a crystal structure based on tetrahedral contacts with only four nearest neighbors. Most of the examples (e.g., diamond) are substances with high melting points.

Distortion of the tetrahedral structure also occurs in the crystalline hydrates of nonpolar solutes. In this case the distortion serves to make room for solute molecules instead of nonbonded water molecules. The structure of one such hydrate, $Cl_2 \cdot 8\ H_2O$, has been determined in detail by X-ray crystallography (Pauling and Marsh, 1952) and is shown in part in Fig. 5-2. The basic element of the structure is a pentagonal dodecahedron. The dodecahedra are arranged (together with six interstitial water molecules per four dodecahedra) so as to define tetrakaidecahedral as well as dodecahedral cavities. The O—H···O distance is 2.75Å, and O—O—O bond angles range from 106 to 125°. A chlorine molecule is too large to fit into the dodecahedral spaces, but it is readily accommodated by the larger tetrakaidecahedral cavities. The calculated composition based on the assumption that only the latter contain Cl_2 is $Cl_2 \cdot 7\frac{2}{3}\ H_2O$, which is in good agreement with the analytical composition, $Cl_2 \cdot 8\ H_2O$. An interesting feature of the structure is that molecules somewhat smaller than Cl_2 could be accommodated by the dodecahedra as well, and the composition would then be $X \cdot 5\frac{3}{4}\ H_2O$. This is exactly the composition of crystalline xenon and CH_4 hydrates, for which detailed crystal structures are not available.

A similar structural reorganization is observed in the crystalline hydrates of some tetraalkyl ammonium salts. One of these, $(iso\text{-}C_5H_{11})_4N^+F^- \cdot 38H_2O$, has been investigated in detail by Feil and Jeffrey (1961). The organic cations are found to be contained in water "cages" formed by the faces of pentagonal dodecahedra. The distance between nearest-neighbor oxygen atoms ranges from 2.75 to 2.86 Å in different parts of the structure, and the O—O—O bond angle ranges from 91 to 133°.

LIQUID WATER

We are of course interested in the liquid, not the crystalline, state of water. Direct structural information comparable to that for the crystalline state is not available for the liquid state, or it might be better to say that a structure in that sense does not exist in the liquid state, in which there can be order at very short range only. This short-range order, however, might be expected to resemble the short-range order in crystalline states since, as was indicated above, the latter is largely dictated by the properties of the H_2O molecule per se. Indeed, all information that bears on the structure in the liquid state confirms this expectation, and the actual problem in discussing liquid water is to decide between alternative ways in which the local ordering of water molecules in ice might be modified in the melting process. We shall briefly discuss some of the experimental data that are relevant to this question, referring the reader to Eisenberg and Kauzmann (1969) for details and for discussion of other properties, such as the optical and dielectric properties.

X-ray Diffraction

X-ray diffraction of liquid water provides us with a radial distribution function for water molecules in a coordinate system in which the center of one molecule is the origin. The most accurate measurements are those of Narten et al. (1967). They show a very sharp peak in the distribution function at a distance that increases with increasing temperature, shifting from 2.82 to 2.94 Å as the temperature goes from 4 to 200°C. This peak represents the average distance between nearest-neighbor molecules and indicates, therefore, that nearest neighbors are on the average farther apart in the liquid than they are in ordinary ice, despite the greater overall density of the liquid which, as in the denser polymorphic forms of ice, presumably arises from a decrease in the distance between nonbonded neighbors. The existence of a well-defined nearest-neighbor separation is of course not peculiar to water: a similar parameter can be measured for all liquids. Water, however, differs from most other liquids in the *intensity* of the peak in the distribution function, which tells us how many nearest-neighbor molecules there are. For most simple liquids this number is relatively large (for close-packed spheres there would be 12 nearest neighbors); in water it is only 4.4. This is unequivocal evidence that the local order about a given water molecule remains close to tetrahedral.

At low temperature the radial distribution function contains additional peaks of low intensity at 3.5, 4.5, and 7 Å, but these peaks disappear gradually as the temperature is raised. At distances of 8 Å or above the positions of water molecules have become completely randomized even at 4°C. The separations of 4.5 and 7 Å are consistent with an icelike tetrahedral model, but the separation of 3.5 Å is not. It provides one of the focal points for attempts to define the difference between short-range organization in the crystalline and liquid states.

Thermochemical Data

The heat of sublimation of ice at 0°C is 11,200 cal/mol. Of this amount, 1400 cal/mol represent the translational enthalpy of the vapor, that is, the kinetic energy of the random motion of the molecules in the gaseous state. The remaining 9800 cal/mol represent the energy required to break the bonds that hold the crystal together. This is primarily the energy of the hydrogen bonds. Van der Waals energy, between nonbonded molecules, which makes a major contribution to cohesion in organic crystals, is relatively unimportant here because of the great separation (4.5 Å) between nearest neighbors that are not hydrogen bonded. The heat of fusion of ice, 1440 cal/mol, is much smaller than the heat of sublimation, and it is thus evident that most of the cohesive energy residing in the hydrogen bonds of the crystalline state is still present in the liquid state. A simplistic interpretation of the numerical results is that about 15% of the hydrogen bonds in ice are broken in the process of fusion. On the other hand, it is equally likely that the hydrogen bonds in the liquid are simply weaker than those in ice (the increase in the average separation of nearest neighbors suggests this), and that the fraction of broken bonds at any instant is very small.

Liquid water, even in the absence of a nonpolar solute, has an anomalous heat capacity, albeit much smaller than those listed in Tables 4-1 and 4-2. Cp is 18 cal/mol deg in the liquid state and 9 cal/mol deg both in the crystalline and vapor states. Even this relatively small anomalous heat capacity cannot be explained on the basis of energy states of individual molecules, and must be attributed instead to a temperature dependence of the energy of intermolecular organization. It could represent an increase in the number of broken hydrogen bonds as the temperature is raised, or a shift in equilibrium between two or more discrete configurational states of similar free energy but different enthalpy and entropy, or it could signify that the intermolecular organization is continuously variable, the average state gradually shifting to higher energy as the temperature is raised. It may be noted that the radial distribution function cannot distinguish between these possibilities, since it represents the average positions of molecules over a long time period. The increase in average nearest-neighbor distance at higher temperatures, as noted above, indicates an average weakening of hydrogen bonds, but cannot distinguish between continuous and discontinuous variability in intermolecular distance or energy.

The OH Stretching Vibration

By carrying out infrared or Raman spectral measurements on dilute solutions of HDO in D_2O or H_2O, it is possible to observe the OH or OD stretching vibration in isolation; each is virtually free from the effects of the coupling that occurs between vicinal oscillators of the same fundamental frequency. The vibration frequency is a sensitive function of the O—H· · ·O or O—D· · ·O bond energy and corresponding bond length, and the resolution is high. Frequencies corresponding to icelike hydrogen-bonded OH or OD (measured in ice containing a little HDO) or to nonbonded OH or OD (measured in dilute solutions of HDO in an inert solvent) are represented by narrow bands separated by 300 to 400 cm^{-1}. An important aspect of this measurement is that the process of light absorption occurs on a time scale that is *small* in comparison with the period of molecular displacements leading to the rearrangement of intermolecular organization. One thus obtains an essentially instantaneous signal from each molecule and not an average signal reflecting the various states through which a molecule passes in a given period of time. By this method one can therefore distinguish between a mixture of hydrogen-bonded and nonhydrogen-bonded OH or OD groups, which would be expected to lead to a two-peak absorption spectrum with the relative intensities of the two peaks shifting with temperature, and a continuous range of possible hydrogen-bond energies, which would be expected to lead to a single relatively broad absorption peak lying between the frequencies for icelike and nonhydrogen-bonded groups.

Both Raman and infrared spectroscopy (Fig. 5-3) yield broad absorption bands of intermediate frequency, with a small increase in peak frequency with temperature (Wall and Hornig, 1965; Stevenson, 1968). This result would seem to be inconsistent with the possible existence in liquid water of a mixture of broken hydrogen bonds and of intact hydrogen bonds of undiminished energy. It provides instead strong evidence for the existence of a broad range of possible hydrogen bond distances. On the basis of the known relation between the vibration frequency and hydrogen bond distance, distribution functions for this distance can be calculated from the spectral data, and they agree well with the time-averaged distribution function derived from X-ray diffraction data (Wall and Hornig, 1965).

It should be pointed out that this interpretation of the spectral data is not universally accepted, and arguments against it have been presented by Frank (1970).

Density

One of the properties of liquid water that has been measured with greatest precision is density (or molar volume) as a function of pressure and temperature. The most striking features are that the density of the liquid is nearly 10% greater than the density of ordinary ice, and that the liquid itself has a negative coefficient of thermal expansion near the melting

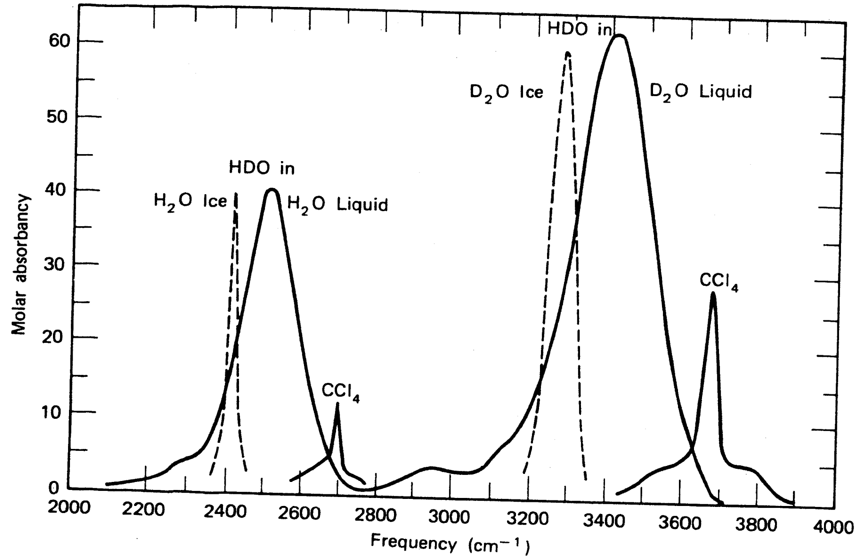

Fig. 5-3. Uncoupled OH and OD stretch bands from infrared spectra of small amounts of HDO in CCl_4, liquid H_2O or D_2O, and crystalline H_2O or D_2O (Stevenson, 1968). From *Structural Chemistry and Molecular Biology*, edited by Alexander Rich and Norman Davidson. W. H. Freeman and Company. Copyright © 1968. (An additional fact of interest is that the vibrational frequencies in CCl_4 are very close to the OD and OH stretching frequencies of HDO in the vapor state.)

point, the density increasing to a maximum at 4°C (for H_2O at 1 atm pressure) before the normal positive thermal expansion takes over. D_2O has a similar density maximum at 11.2°C. This intriguing phenomenon turns out not to be a sensitive discriminant between different ideas concerning the liquid structure, because it is primarily a consequence of the open low-density structure that is imposed on ice by the tetrahedral hydrogen-bonded water molecules. Any process that leads to disruption of this structure and increased randomization of the intermolecular arrangement must increase the density. The usual interpretation of the density maximum in the liquid state supposes that this maximum results from a continuation of the same kind of structural rearrangement that distinguishes the liquid state from the crystalline state, with normal thermal expansion acting in opposition.

The same simple interpretation applies to the volume change associated with the hydrophobic effect. When hydrophobic solutes are introduced into an aqueous solution, they occupy interstitial positions in the framework of hydrogen-bonded water molecules. The distortion of this framework that is then required can occur without a large increase in volume, and the overall volume change for the addition of the solute (the thermodynamic partial molar volume) is therefore less than it would be for addition to more typical solvents with a less open liquid structure. The anomalous further decrease in volume at low solute concentration that was discussed on p. 19 cannot be accounted for in this way, but requires an additional mechanism for contraction. A possibility is an arrangement whereby water molecules are shared between two adjacent hydrocarbon chains.

MODELS FOR LIQUID WATER

The properties of liquid water just discussed and other physical properties do not permit an unequivocal interpretation of the structure of liquid water. The most attractive model is probably the distorted hydrogen-bond model of Pople (1951), recently extended by Bernal (1964), and this is the model favored by Eisenberg and Kauzmann (1969). It is conceptually a very simple model. It assumes that all water molecules in the liquid continue to be hydrogen bonded to four neighboring water molecules, but that the intermolecular links can be bent and stretched to produce irregular and varied networks. We already know that such distorted networks exist from our examination of the crystal structures of the denser forms of ice and the crystalline gas hydrates, whereas there are no known crystal structures containing broken hydrogen bonds. Distorted and weakened hydrogen bonds are consistent with the spectroscopic data, which seem to exclude the possibility of a significant fraction of broken hydrogen bonds. The model originally formulated by Pople (1951) does not account for the 3.5 Å peak in the radial distribution function obtained by X-ray scattering, but the extension by Bernal (1964) can do so. Second nearest-neighbor water molecules, not hydrogen bonded to the central water molecule of the coordinate system, lie at a distance of about 3.5 Å from the central molecule in several of the high-density crystalline forms of ice.

Many workers who have attempted to account for the properties of liquid water in terms of a structural model have done so in terms of mixture models. There exist at least a dozen such models, all of which share the basic premise that liquid water is a mixture of several distinct molecular species. All of these models require the existence of broken hydrogen bonds: in some models they occur at the boundaries of postulated molecular clusters of different kinds, and in others they arise through the postulated presence of free gas like H_2O molecules in the interstices of hydrogen-bonded networks. By assigning appropriate energy and density parameters to the different molecular species, one is able to evaluate the equilibrium distribution among the several species as a function of external conditions, and to calculate the overall thermochemical properties and the density as a function of, for example, temperature. The claim to validity of a particular model then rests on the often close agreement between such calculations and the corresponding experimental data. Many of the parameters that enter into such a calculation are, however, unknown, and are arbitrarily adjusted to bring this close agreement about. In some instances the number of adjustable parameters equals or exceeds the number of parameters in a purely empirical equation that can be used to fit the data without reference to any structural considerations at all. Under these circumstances the claim to validity based on agreement between theory and experiment loses most of its force, and is not able to counterbalance the principal experimental arguments against mixture theories, which are (a) that they have no counterpart among the known structures of the various forms of crystalline ice, and (b) that they seem to be in conflict with spectroscopic data. Nevertheless, mixture theories have enthusiastic advocates, and Frank (1970), for example, has presented a vigorous defense of them against the arguments raised by infrared and Raman spectral data.

One of the most highly developed theories of the structure of liquid water is the mixture theory of Némethy and Scheraga (1962). It has the advantage of great simplicity: variations in configurational energy are considered as arising entirely from the *breaking* of hydrogen bonds. In attempting to fit this theory to experimental thermochemical data, the authors were compelled to adjust their parameters in order to produce what is surely an unacceptably large fraction of broken hydrogen bonds (greater than 50%) and a remarkably low value for the energy required to break a hydrogen bond, which turns out to be only 1.3 kcal/mol. How small this figure is may be seen from the estimate made by Scheraga (1961) in another place, that the energy of breaking a hydrogen bond should normally be about 6 kcal/mol. The figure of 1.3 kcal/mol is in fact closer to the energy required to *distort* a hydrogen bond (a few tenths of a kcal/mol to judge from the enthalpies of transition between various crystalline forms of ice) than to the energy required to *break* a hydrogen bond. One way to look at this result is to consider it as additional evidence in favor of the distorted bond model.

It would clearly be desirable to use a model for water consistent with the distorted bond arrangement that direct structural studies suggest, or, better still, to build a structural model on the basis of molecular interaction parameters, from which the existence of distorted rather than broken hydrogen bonds would emerge as a predicted result. A model based explicitly on a distribution function for hydrogen-bond orientation has in fact been put forward, and it treats aqueous solutions of hydrophobic solutes as well as water itself (Marčelja et al., 1977). Approximations in the mathematical analysis are numerous, so that it is difficult to assess whether any new insight is gained. Perhaps of greater interest is a theory based on specified interaction potentials between water molecules (Rahman and Stillinger, 1971, 1972, 1973). A computer simulation is used to allow an assembly of water molecules to find its own preferred arrangement within the constraints of the programmed interaction potentials, that is, explicit relations between interaction potentials and macroscopic observables are not used. One of the results obtained is that closed nonshort-circuited networks containing as many as 12 hydrogen-bonded water molecules (e.g., as in Fig. 5-2) can be expected to exist even in pure water. There have been many other theoretical approaches, and they are summarized by Ben-Naim (1974) and Franks (1975).

In a paper specifically addressed to the hydrophobic effect, Pratt and Chandler (1977) use explicit potential functions for interaction between hydrocarbon molecules and between hydrocarbon and water, but not for the interaction of water molecules with each other, which are described empirically in terms of experimental distribution functions derived from X-ray scattering data for pure water (Narten and Levy, 1971). One motivation for such a semiempirical approach is stated to be the present "unavailability of any microscopic theory for the structure of fluids with strong attractive interactions. The current state of the art allows us to study the effects of strong repulsive interactions and weak attractive intermolecular forces. For most liquids they are the only kinds of interactions." But for water they are not.

It is debatable whether any of the theoretical investigations published so far do more than restate in somewhat different language what is already known on an empirical basis. As Kauzmann (1976) has pointed out, no present theory can explain the effect of pressure on the properties of water. As for the process of chief interest in this book, the assembly of simple amphiphiles into small micelles, of phospholipids into bilayers, and of proteins and lipids into functioning biological membranes, it is difficult to see any contribution that the theories can make to their understanding. As the following chapters will show, these complex phenomena can be semiquantitatively predicted from the experimental data for the hydrophobic effect which we have cited, without the necessity for a detailed model of the structure of the solvent.

STRUCTURAL CHANGE ASSOCIATED WITH THE HYDROPHOBIC EFFECT

At the risk of being repetitious, a summary of what may occur when hydrocarbon chains are dissolved in water is given here. The crystal structures briefly discussed in this chapter provide a frame of reference that may be used as a point of departure. They suggest that water molecules are readily ordered into networks forming cagelike cavities within which nonpolar solutes may be enclosed, and that this may be done without breaking hydrogen bonds or altering the intrinsic coordination pattern of four nearest-neighbor water molecules. The cavity volume is apparently readily variable. The simplest gas hydrates (Fig. 5-2) have dodecahedral cavities large enough to contain Xe or CH_4, and somewhat larger tetrakaidecahedral cavities. In the crystalline hydrate of tetra(isoamyl) ammonium fluoride (Feil and Jeffrey, 1961) an even larger pentakaidecahedral cavity is observed. There is no reason to believe that these two known crystal structures exhaust the possible size range.

If such structures exist around nonpolar molecules in the liquid state, they are likely to be imperfect: random distortions leading to disorder are likely to be superimposed on the uniquely ordered bond distortions that lead to formation of polyhedral cages. The likelihood of such a process is suggested first by analogy: randomness certainly occurs in pure liquid water, since the time-average order as given by the radial distribution function extends to only 8 Å. Second, however, it must be kept in mind that an ordered array of water molecules around a nonpolar solute in a dilute solution is itself surrounded by pure liquid water, and that most water molecules in the postulated polyhedral cages will be hydrogen bonded to an external water molecule as well as to each other. The ordered structure is thus likely to be influenced by the rapidly fluctuating structural changes around it.

The likely occurrence of imperfections and fluctuations makes it not unreasonable that a hydrocarbon chain that is part of an amphiphilic molecule, and thus unable to generate a completely closed solvent cage, should nevertheless have thermodynamic properties similar to those of a complete hydrocarbon molecule in water solution. One can also readily visualize within the range of possibilities suggested by these considerations why the anomalous heat capacity associated with the hydrophobic effect is so large, and why the partial molar entropy and volume are negative.

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SIX

MICELLES

INTRODUCTION

When amphiphilic molecules are dissolved in water they can achieve segregation of their hydrophobic portions from the solvent by self-aggregation. The aggregated products are known as *micelles*.[1] When the hydrophobic portion of the amphiphile is a hydrocarbon chain, the micelles will consist of a hydrocarbon core, with polar groups at the surface serving to maintain solubility in water. Micelles can be small spheres or disks, oblate or prolate ellipsoids, or long cylinders. They can also be in the form of bilayers, that is, two parallel layers of amphiphile molecules with the polar groups facing out. Bilayer micelles often form more or less spherical vesicles with an internal solvent-filled cavity.

The hydrocarbon chains in such micelles are generally regarded as disordered, so that the hydrophobic core is in effect a small volume of *liquid* hydrocarbon. This differs from a large volume of liquid hydrocarbon only by the restrictive influence of the close proximity of all parts of the liquid to the surface, as a result of which, for example, parts of hydrocarbon chains closest to the polar groups are constrained to be more or less perpendicular to the surface. The effects of such constraints are, however, quite small, at least for micelles formed from simple amphiphile molecules. This can be shown both by spectroscopic relaxation methods, which measure the freedom of motion of hydrocarbon chains in the micelle, and by spectroscopic and thermodynamic studies of small solutes dissolved in the micelle, which behave remarkably as they would in a liquid hydrocarbon medium of much greater extent. For some of the more complex amphiphiles, a temperature above room temperature is required for the existence of disordered micelles.

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[1] There is no universally accepted definition of the term *micelle* and some workers would not include aggregates based on a bilayer structure within the definition. The term is used here to designate any water-soluble aggregate, spontaneously and reversibly formed from amphiphile molecules or ions. As will be seen, the most stable form of such aggregates is dictated by thermodynamic factors, and the conditions under which globular aggregates or bilayer structures will be formed are readily predictable.

The simplest evidence for the liquidlike nature of the micelle interior comes from the ability to dissolve hydrocarbons and other hydrophobic substances within them. One quantitative study leading to precise thermodynamic parameters for this process has been carried out by Wishnia (1963), who measured the solubilities of hydrocarbons in sodium dodecyl sulfate micelles, in 0.1 M NaCl as overall solvent. The micelles in this case (from measurements to be cited later) contain on the average about 85 amphiphile ions per micelle (at 25°C), and they are probably disklike in shape. However, this information is actually not necessary for estimation of thermodynamic parameters, since the mole fraction (X_{mic}) of solute in the micelles can be obtained by knowing only the total number of dodecyl sulfate ions in micellar form and the added solubility of hydrocarbon (above that in 0.1 M NaCl alone) that they produce. Assuming that the hydrocarbon within the micelle constitutes an ideal solution, we can obtain the standard free energy of transfer to the micelle interior in the same way as for other solubility measurements, that is,

$$\mu^{\circ}_{\text{mic}} - \mu^{\circ}_{W} = RT \ln (X_W/X_{\text{mic}}) \tag{6-1}$$

Measurements were made at several temperatures from 15 to 35°, and heats and entropies of transfer were obtained as outlined in Chapter 4. Unfortunately the data were not of sufficient precision to permit the evaluation of the change in heat capacity. The results obtained are summarized in Table 6-1. A least-squares treatment of the free energies of transfer leads to the relation (in cal/mol)

$$\mu^{\circ}_{\text{mic}} - \mu^{\circ}_{W} = -\ 1934 - 771\, n_{\text{C}} \tag{6-2}$$

The experimental uncertainty is relatively large, $\pm$ 160 cal/mol in the constant term and ± 43 cal/mol in the slope.

These results are to be compared with the corresponding data for transfer to a liquid hydrocarbon, as given by equation 2-3 and in Table 4-1, the latter with signs reversed. The similarity is striking. The free energies of transfer are smaller by about 15%, and this difference stems largely from a slightly smaller entropy of transfer. The enthalpies are nearly the same (subject to an experimental error of the order of 500 cal/mol), and even the change in sign with increasing chain length is duplicated. The small difference in entropy undoubtedly reflects the previously men-

Table 6-1. Thermodynamics of Transfer from Water to the Interior of a Dodecyl Sulfate Micelle[a]

	$\mu^\circ_{\text{mic}} - \mu^\circ_W$ (cal/mol)	$\bar{H}^\circ_{\text{mic}} - \bar{H}^\circ_W$ (cal/mol)	$\bar{S}^\circ_{\text{mic}} - \bar{S}^\circ_W$ (cal/deg mol)
Ethane	−3450	+2000	+18.3
Propane	−4230	+1000	+17.5
Butane	−5130	0	+17.2
Pentane	−5720	−1100	+15.6

[a] From Wishnia (1963). Results are for 25°C.

tioned constraints within the micelle that result from the necessity for some degree of orientation near the surface, and the difference is actually surprisingly small. It is clear from these data that the core of a dodecyl sulfate micelle must represent a medium that is very close indeed to a liquid hydrocarbon.

SPECTROSCOPIC RELAXATION METHODS

Spectroscopic relaxation methods have proved to be very useful for estimation of fluidity in micelles and other systems, in terms of the rate of motion of molecules within the systems. All such methods must satisfy two criteria: (1) they must be able to resolve spectral differences that arise from the geometrical orientation of the absorbing entity in an electromagnetic field, and (2) the time scale of the measurement must be comparable to the time scale of the molecular motion one wishes to observe, so that orientational differences are averaged out if molecular motion is fast, but remain observable if motion is slow. Free rotational motion of *small molecules* in a medium of *low viscosity* occurs with a relaxation time of the order of 10^{-8} sec, and this therefore defines the required time scale of the experiment. Relaxation processes for larger molecules or for particles such as micelles occur more slowly, and one can therefore distinguish between motion of a molecule (or a portion of a molecule) within the micelle and motion of the micelle as a whole. An increase in the viscosity of the medium also slows down the relaxation process, and formation of an ordered phase will abolish some motions entirely, so that the state of the local environment near the molecule that is being observed can be investigated.

One method satisfying these criteria is *fluorescence depolarization* (for an introduction with reference to basic source material, see Stryer, 1968).

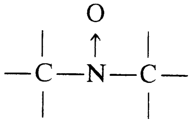
The lifetime of the excited state in fluorescence measurements is of the order of nanoseconds, and dependence on geometrical orientation can be readily introduced by using polarized light for excitation, so that in a liquid medium chromophores with transition moments parallel to the plane of polarization will be preferentially excited. In the absence of molecular motion, the emitted fluorescent radiation will be partially polarized, reflecting the preferred orientation of the emitters. Molecular motion within the lifetime of the fluorescent state will result in diminution of the extent of polarization. The most straightforward procedure is to use a pulse of nanosecond duration for excitation and then to follow the polarization of the fluorescence as a function of time for as long as it can be observed. If the molecular motion is isotropic (no preferred axis for rotation), it is characterized by a single relaxation time τ, with the result that the degree of polarization at time t, $P(t)$ is given by

$$P(t) = P(0)e^{-3t/\tau} \tag{6-3}$$

where $P(0)$ is the degree of polarization at some suitably defined zero time. If the motion is anisotropic there would be more than one exponential term on the right-hand side of equation 6-3. A less direct method is to use steady state measurements, that is, to measure the polarization of fluorescence under constant excitation. This is essentially equivalent to measurement of the molecular motion at a single time determined by the lifetime of the excited state, and requires assumptions about whether the motion is isotropic or not. If equation 6-3 is deemed applicable, $P(0)$ must be determined independently, and this is normally done by varying the viscosity of the medium and extrapolating to infinite viscosity, a procedure that may not always be valid.

Since hydrocarbon chains are not fluorescent at usable wavelengths, the micelle interior may be investigated by use of *fluorescent probes*, which are molecules containing suitable chromophores that are soluble in the micelle. That a given probe is actually dissolved in the hydrophobic core can be readily established since the characteristic absorption and emission spectra are usually significantly affected by the polarity of the medium: some, like the frequently employed probe 1-anilino-8-naphthalene sulfonate, display virtually no fluorescence in water. Shinitzky et al. (1971) have used two aromatic hydrocarbon probes of this kind to investigate micelles formed by several cationic amphiphiles, employing the steady state method. The relaxation times observed were found to be too fast to be ascribable to motion of the micelle as a whole and depended little on micelle size. They were somewhat slower, however, than relaxation times observed for the same chromophores in pure liquid hydrocarbons of appropriate chain length. Both from the relaxation times themselves and from their dependence on temperature, they concluded that the micelle interiors are liquid, albeit with a higher viscosity than a pure hydrocarbon such as dodecane would exhibit.[2] This higher viscosity is to be expected, since the hydrocarbon chains of micelles are anchored to the surface hydrophilic groups, and thus restricted in their motility. The same effect is seen in many other systems: for example, liquid aliphatic alcohols have a viscosity several times higher than the corresponding liquid hydrocarbons, reflecting the existence of networks of hydrogen bonds between alcohol OH groups, that constrain the motion of one end of the hydrocarbon chain.

A second procedure is to use the electron spin resonance (esr) spectrum observable for free radicals in a magnetic field (Hamilton and McConnell, 1968; Griffith and Waggoner, 1969). Since the systems of interest do not contain free radicals, the use of a probe substance is again required. The dialkyl nitroxide radical



$$-\underset{|}{\overset{|}{\mathrm{C}}}-\overset{\overset{\mathrm{O}}{\uparrow}}{\mathrm{N}}-\underset{|}{\overset{|}{\mathrm{C}}}-$$

has been found to be especially useful, since it is remarkably stable and unreactive, and can be readily incorporated into a variety of organic molecules. The resonance time is again of the order of 10^{-8} sec, and the geometrical factor here is provided by the unique direction associated with the spin transition: the hyperfine splitting seen in the esr spectrum is resolvable into two components ($A_{\parallel}$ and $A_{\perp}$), one corresponding to the transition moment parallel to the applied magnetic field, the other to perpendicular orientation. The characteristic positions and intensities for a given probe can be determined by use of crystalline or otherwise ordered systems. Molecular motions that occur more rapidly than 10^{-8} sec result in sharp resonance lines that represent the *time average* of all geometric states encountered during the resonance period. In a liquid, where motion is completely isotropic, only a single value for A is therefore observed. On the other hand, if interchange of geometrical states is slow, both components will be observed. Rapid *anisotropic* motion (e.g., torsional motion about a fixed molecular axis) results in observation of both components, but with characteristics different from those of a crystalline sample. In that case, the difference between the observed values for $A_{\parallel}$ and $A_{\perp}$ and those in the crystalline reference can be used to determine the average orientation resulting from the rapid motion, since there is a simple relation between the observed value of A and the contributions of the reference values to it. Line width and shape are also helpful in the analysis of local modes of motion, as in nuclear magnetic resonance (see below).

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[2] Rotational diffusion constants are inversely proportional to the viscosity of the medium, and an apparent viscosity [termed *microviscosity* by Shinitzky et al. (1971)] can be obtained either by measuring τ for the same probe in liquids of different viscosities or, if the size and shape of the probe molecule can be established, by the use of equations relating size, shape, and solvent viscosity. Shinitzky et al. obtained microviscosities of 17 to 32 CP at 27° for the micelle interior, when using amphiphiles with alipathic hydrocarbon chains of 12 to 16 carbon atoms. These values are to be compared with viscosities of 3.0 CP for liquid n-hexadecane at a comparable temperature, and of 13.4 CP for n-hexadecyl alcohol at 50°C (about twice this value is to be expected at 25°C).

Nitroxide free radical probes introduced into sodium dodecyl sulfate micelles do not show separate components in the esr spectrum, thus indicating that they are in a liquid medium, undergoing isotropic motion (Waggoner et al. 1967). The spectral lines are somewhat broader, however, than they are in aqueous solution indicating, as did the fluorescence measurements discussed previously, that the local viscosity is fairly high. More extensive use of the technique has been made for micelles formed by biological lipids, and these results are discussed in Chapter 12. Some of these experiments have been done with the nitroxide free radical covalently attached to specific carbon atoms of the lipid hydrocarbon chains, and the esr spectra then indicate that the segments of a hydrocarbon chain nearest the amphiphile head group will generally (i.e., in micelles formed by simple amphiphiles, as well as those formed by biological lipids) be more restricted in their motion than more distal parts of the chain, as is to be expected. In other words, the relatively high viscosity of the liquid interior of a micellar structure, as observed by dissolved fluorescent or free radical probes, is an average property. Methods that can define resistance to motion for volume elements of atomic dimensions (the use of the term *viscosity* becomes increasingly artificial with such localization) show that the micelle interior is not homogeneous with respect to this property.

The use of an added probe (whether free or covalently attached) has a major disadvantage in that the probe, having a size comparable to that of the localized volume we wish to study, is likely to perturb the system. This difficulty can be overcome by using high resolution nuclear magnetic resonance (nmr) spectroscopy, by which nuclei that are constituents of the hydrocarbon chain itself can be studied. Initial application of this technique involved proton magnetic resonance (pmr), which suffers from the disadvantage that the hydrogen nucleus is not affected by interactions of very short range, but only by relatively long-range interactions with other nuclei, chiefly other protons. These interactions can be intermolecular as well as intramolecular, and are often too numerous to be resolved. The method can always give a qualitative result: if all motions are rapid enough to permit time-averaging of all such interactions within the resonance period (again about 10^{-8} sec), a sharp absorption line will be obtained; if motion is slow or restricted, line broadening will be observed, sometimes to the extent that a line will not be visible at all against background noise. A quantitative interpretation of line broadening in intermediate situations is usually difficult. Waggoner et al. (1967) have shown that line widths in the pmr spectrum of the CH_2 and CH_3 groups of dodecyl sulfate micelles in water are as narrow as those observed in liquid dodecanol, showing that their freedom of motion is also the same. They also dissolved a nitroxide free radical probe in the micelles and, in addition to obtaining the esr spectrum of the probe itself, as discussed above, determined its effect on the line width of the CH_2 signal in the pmr spectrum. A broadening is observed, arising from the added spin–spin interaction, and it was found to be quantitatively consistent with a calculation based on free rapid motion of the probe in the micelle interior.

^{13}C-nmr suffers from the same disadvantage. The spectra are dominated by proton–carbon dipolar interactions, which, though not as numerous as proton–proton interactions, are still too numerous for unambiguous analysis. A summary of results obtained by ^{13}C-nmr is provided by Lee et al. (1974) and by Wennerstrøm and Lindblom (1977).

On the other hand, there is considerable gain in the use of deuterium magnetic resonance (dmr) (Seelig, 1977), ascribable to the fact that the ^2H nucleus has a nuclear spin of 1, giving rise to a quadrupole moment that can interact with an *electric* field gradient in its vicinity. (^1H and ^{13}C have a nuclear spin of $\frac{1}{2}$ and possess only a magnetic dipole moment, which interacts strongly only with other *magnetic* dipoles.) The dominant interaction is with the electric field of the bond by which the deuterium atom is attached to the rest of the molecule, which for a deuterated hydrocarbon would be the C—D bond, and the spectrum is extremely sensitive to the orientation of this bond in the magnetic field. At the same time the magnetic dipole moment of ^2H is small, and interactions with vicinal proton dipoles have little effect on the spectrum. The dmr spectrum therefore becomes as easy to interpret as the esr spectrum of a nitroxide probe. In the simplest case (one CD_2 group in an otherwise protonated hydrocarbon chain), the spectrum consists of a single doublet, the separation between the two peaks being a measure of the rate and anisotropy of motion in the same way as described above for the $A_{\parallel}$ and $A_{\perp}$ components of the nitroxide esr spectrum. The advantage of using dmr rather than a nitroxide radical is of course that selective replacement of D for H is not expected to perturb the natural arrangement of the hydrocarbon chains. A disadvantage in the use of dmr is that the spectral intensity is low, so that high concentrations and high efficiency of D for H substitution are required. It should also be noted that the intrinsic resonance frequency is about 100 times smaller for dmr than for pmr and esr, so that motions on a time scale of 10^{-8} sec would be averaged out in dmr, whereas separate signals are still observable when the other techniques are used.

Because of the concentration requirement, dmr measurements have not been reported for small soluble micelles, but they have been made for ordered bilayers (with intervening layers of water) that can be formed by simple amphiphiles at very high concentration. For example, a mixture of Na dodecanoate (32 wt. %), decanol (38 wt. %), and water (30 wt. %) has been used. Specifically deuterated forms of both the fatty acid and the alcohol were synthesized, with CD_2 groups at various positions on the hydrocarbon chain, and measurements were made on bilayers containing each deuterated species in turn (Seelig and Niederberger, 1974). Even in this system, which must be more constrained than a small micelle because it consists of tightly packed parallel bilayers, the hydrocarbon core was found to be fluid. Freedom of motion was found to increase with distance from the amphiphile head group, as is also observed by the spin label method, but the results obtained by the two methods differ in detail, presumably because of the perturbing effect of the bulky spin label in the esr experiments. The dmr results indicate that the first six CH_2 groups closest to the amphiphile head group have about equal "order parameters" (reflecting limitation on motional freedom), and that disorder increases more or less linearly beyond that.

The overall conclusion from all experiments of this kind is that the liquid state in the hydrocarbon core of a micellar assembly is appreciably different from the bulk liquid state. (1) The hydrocarbon chains are unable to tumble, and, on the average, remain oriented perpendicular to the hydrocarbon–water interface. (2) In such an array of more or less parallel chains, isolated *gauche* connections as in Fig. 6-1*B* are improbable[3], the random fluctuations that must be present in any fluid state are much more likely to be "kinks" of the kind illustrated in Fig. 6-1*C*, which leave the overall direction of the chain unchanged. (3) In the bilayer arrangement, where amphiphile head groups are tightly packed into a planar layer, even "kinks" do not occur in the six bonds closest to the head group. This restriction may not apply to small micelles with curved surfaces, where the separation between head groups is much larger (see below).

[3] The requirement that all connections must be *gauche* or *trans* stems from the restrictions on rotation about single bonds of a hydrocarbon chain (Flory, 1969). For additional theoretical discussion, see Schindler and Seelig (1975).

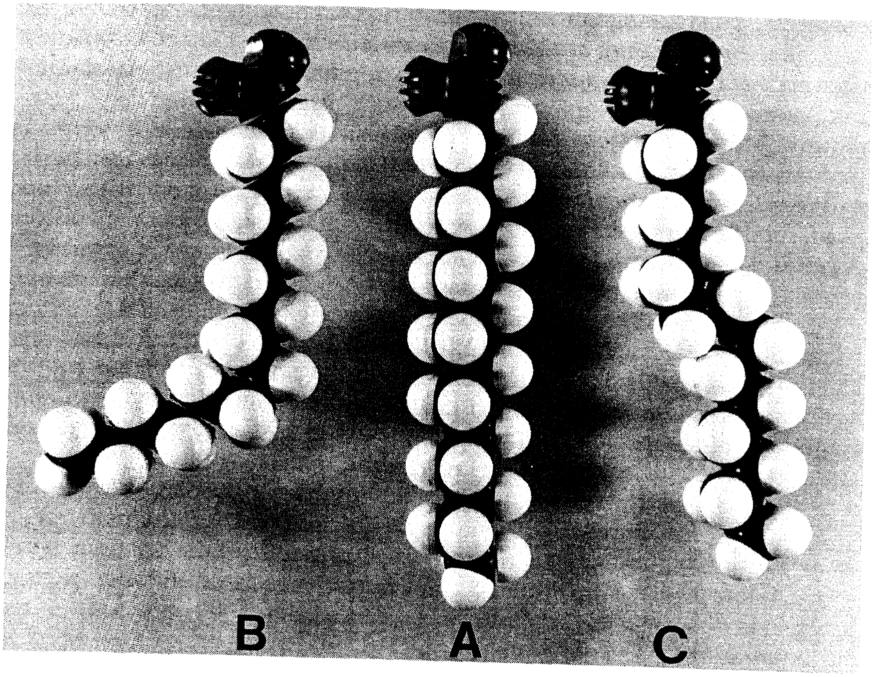

Fig. 6-1. (*a*) Hydrocarbon chain in the all-*trans* configuration, as it would exist in the crystalline state. (*b*) Hydrocarbon chain with one *gauche* connection in an otherwise all-*trans* chain. (*c*) Two *gauche* connections in opposite directions, creating a "kink." (Based on Seelig, 1977.)

CRYSTALLIZATION OF VERY LONG HYDROCARBON CHAINS

The foregoing data have shown that amphiphiles with ionic head groups and hydrocarbon tails of up to 16 carbon atoms form micelles with essentially liquid cores at room temperature. It is important to note in this connection that pure saturated hydrocarbons form crystalline solids that melt below room temperature only when $n_C < 18$, as shown by the data of Table 6-2. The crystal structure is based on parallel extended hydrocarbon chains in the all-*trans* configuration, and the packing differs between molecules with odd and even values of n_C, as is reflected in the alternation of thermodynamic properties seen in Table 6-2. Because hydrocarbon chains are necessarily bent at an angle where double bonds occur, unsaturated hydrocarbons form less stable crystals than saturated ones, which melt well below room temperature for all chain lengths of interest.

Table 6-2. Thermodynamics of Melting of Crystalline Hydrocarbons[a]

	Melting Point (°C)	ΔH° (kcal/mol)	ΔS° (cal/deg mol)	ΔG° at 25°C (kcal/mol)
$C_{13}H_{28}$	− 5.4	6.8	25.4	−0.77
$C_{14}H_{30}$	5.9	10.8	38.6	−0.74
$C_{15}H_{32}$	10.0	8.3	29.2	−0.44
$C_{16}H_{34}$	18.2	12.8	43.8	−0.30
$C_{17}H_{36}$	22.0	9.7	32.8	−0.10
$C_{18}H_{38}$	28.2	14.8	49.2	+0.16
$C_{19}H_{40}$	32.0	12.0	36.0	+0.25
$C_{20}H_{42}$	36.7	16.8	54.2	+0.63

[a] Data are for saturated *n*-paraffins based on compilations by the American Petroleum Institute (1953). Values of ΔH° and ΔS° refer to the melting point. Values of ΔG° at 25°C are calculated assuming constant ΔH° and ΔS° between 25°C and the melting point. Crystals formed by unsaturated hydrocarbons melt at much lower temperatures.

These data suggest that micelles formed by amphiphiles with very long saturated hydrocarbon chains may have an ordered hydrocarbon core at room temperature, which becomes liquidlike only at higher temperatures. Moreover, where head group interactions favor ordering of the hydrocarbon chains, the transition from ordered to liquidlike core may occur at higher temperatures than the melting points of pure hydrocarbons of comparable chain length. Micelles formed from amphiphiles containing unsaturated hydrocarbon chains would be much less likely to contain ordered structures.

It will be seen in Chapters 11 and 12 that ordered arrangements of hydrocarbon chains, with appropriate melting temperatures, do in fact occur, as predicted, in phospholipids. They are not of interest in relation to the formation of small soluble micelles by simple amphiphiles, and the discussion in this and the two following chapters will be confined to micelles with liquid hydrocarbon cores.

GEOMETRIC CONSIDERATIONS

Micelles formed by simple amphiphiles can be viewed as consisting of a core of liquid hydrocarbon, surrounded by a mantle containing the amphiphile head groups. It will be assumed that water molecules are excluded from the hydrocarbon core because the avoidance of H_2O-hydrocarbon contacts is the basis for the self-association process. Water molecules can and do, however, penetrate the micellar mantle, and some H_2O-hydrocarbon contacts at the interface between the core and the mantle are inevitable. The number of such contacts is determined by the surface area of the hydrocarbon core, and this area is also a measure of the separation between head groups in the mantle. Surface area thus becomes the critical factor in the thermodynamics of micelle formation. It therefore becomes important to discuss the geometry of micelles and the purely geometrical constraints that affect the surface area. These may be summarized as follows (Tartar, 1955; Tanford, 1972):

1. Although the liquid state in the micelle core is not the same as in bulk liquid hydrocarbon, this should have little effect on molecular volumes. Measured volumes based on X-ray scattering data for micellar aggregates (Reiss-Husson and Luzzati, 1964) are in fact essentially the same as volumes based on published densities of liquid hydrocarbons or on the empirical volume additivity rule of Traube (1899) (discussed by Cohn and Edsall, 1943, p. 147). The following general relation may be written for the core volume V of a micelle containing m' saturated hydrocarbon chains:

$$V = m'\,(27.4 + 26.9\,n'_C) \tag{6-4}$$

where V is in cubic angstroms and n'_C is the number of carbon atoms per chain that are embedded in the micelle core. As noted earlier (p. 14), the numerical value of n'_C may be one less than n_C, the total number of carbon atoms per chain. For simple amphiphiles with a single alkyl chain, m' is the same as the number of monomer molecules per micelle (m), but we shall also apply equation 6-4 to molecules containing two hydrocarbon chains, in which case $m' = 2m$.

2. Since no hole can exist at the center of the micelle, one dimension is always limited by the maximum possible extension of hydrocarbon chain. This distance is obtained from the distance of 2.53 Å between alternate carbon atoms of a fully extended chain, with the addition of the van der Waals radius of the terminal methyl group (2.1 Å) and one-half the bond length to the first atom not contained within the hydrophobic core ($\simeq$0.6 Å). The maximum length $l_{\max}$ for a chain with n'_C embedded carbon atoms, in angstroms, becomes

$$l_{\max} = 1.5 + 1.265 n'_C \tag{6-5}$$

Hydrocarbon chains in the liquid state are not fully extended, so that the effective length of a chain (perpendicular to the micelle surface), which we shall call b, is less than $l_{\max}$. In bulk liquid the difference can be estimated on the basis of the statistical mechanics of polymer chain dimensions (Flory, 1969). For a chain of typical lenth ($n'_c = 11$), one would estimate $b/l_{\max} = 0.75$ at room temperature (Tanford, 1974). In the more ordered state of the micelle, each "kink" of the type illustrated by Fig. 6-1*C* would reduce the chain length by 1.25 Å. For two kinks per chain, $b/l_{\max}$ would be about 0.80 for a typical chain length.

3. For an enclosed volume of any assumed shape, the surface/volume ratio *always decreases* as the volume increases. Since the volume is proportional to m' (equation 6-4), this means that the surface area per hydrocarbon chain (A'), averaged over the entire surface, must decrease as the micelle size increases: for spherical micelle cores, for example, A' would be proportional to $(1/m')^{1/3}$. For simple amphiphiles with a single hydrocarbon chain per molecule, A' is equal to A, the average area per molecule, which is also the area per head group. For molecules with two alkyl chains attached to the same head group, $A = 2A'$.

4. The fixed bond lengths and angles of a hydrocarbon chain impose restrictions beyond the already-mentioned limitation on one dimension of the micelle core. For example, hydrocarbon chains at the point of emergence from the micelle core cannot be arbitrarily distorted to create a perfectly smooth interface between core and mantle. Within the confines of a model that treats the core interior as a continuum, the surface has to be imagined as "rough" (Fig. 6-2), with peaks and troughs that would presumably be in a state of fluctuation if the micelle core is liquidlike. The smooth surface would then represent a time-averaged surface, but the area of contact with solvent at any instant would be a few percent larger. Further restrictions of this kind are considered by Israelachvili et al. (1976), but they are probably relatively unimportant in terms of the crude continuum model for a micelle used here.

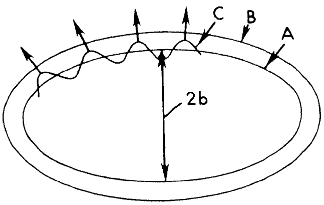

Fig. 6-2. Schematic illustration of surface roughness. A is the smooth interface between micelle core and mantle, which would be calculated for a given volume of hydrocarbon if one could treat the latter as a structureless fluid, and B is the corresponding surface for the location of head groups. The rough surface (C) extends beyond A at the points of emergence of head groups, which are shown by arrows. The total volumes enclosed by surfaces A and C must be the same.

SMALL COMPACT MICELLES

Micelles formed by simple amphiphiles are often quite small, with an aggregation number of the order of 100 for molecules with a dodecyl hydrocarbon chain, and even lower when the alkyl chain is shorter. Even such small micelles are, however, too large to be accommodated within a spherical shape, given that the radius of a spherical micelle core is limited, as discussed above. For example, the weight-average aggregation number ($\overline{m}_w$) of dodecyl sulfate micelles ranges from 62 in the absence of added salt to 126 in 0.5 *M* NaCl (Mysels and Princen, 1959; Emerson and Holtzer, 1967). The maximum number for a spherical micelle depends on the choice of a value for n'_C, but even with $n'_C = n_C = 12$ the maximum value of m is only 23 if we set $b = 0.75\, l_{\max}$ and still only 56 if we allow $b = l_{\max}$. It is probably more realistic to set $n'_C = 11$ for a dodecyl chain, and the maximal aggregation number for a sphere is then even smaller.

To increase the volume of the micelle core, with one dimension (b) remaining fixed, there are basically only two simple possibilities: a disklike shape ($a \times a \times b$) or a rodlike shape ($a \times b \times b$), where a is the dimension that can grow to any length. Figure 6-3 illustrates two examples of each, but many other shapes are possible (Israelachvili et al., 1976). Because of the liquid nature of the micelle core, and the likely fluctuating roughness of its surface, it may be unrealistic to think in terms of a precise shape, such as a rigid body would have, and there may not be a better description than "disklike" and "rodlike." Regardless of the exact choice of geometrical model, the surface-to-volume ratio decreases more steeply with increasing aggregation number for disklike than for rodlike micelles, because the former have two variable dimensions and the latter only one.

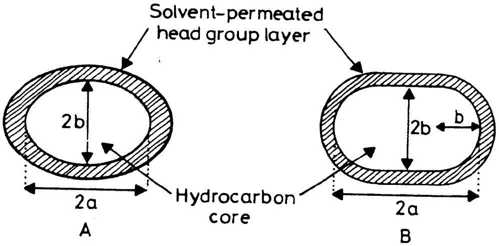

Fig. 6-3. Cross-sectional representations of nonspherical micelles, with b designating the limiting length of an amphiphile hydrocarbon chain. (*a*) Ellipsoidal micelles: rotation of the ellipse about the minor axis (2*b*) leads to an oblate ellipsoid; rotation about the major axis leads to a prolate ellipsoid. (*b*) Rotation about the axis 2*b* leads to a flat disk with a semitoroidal ring abound the edge; rotation about the long axis leads to a cylinder with hemispherical caps.

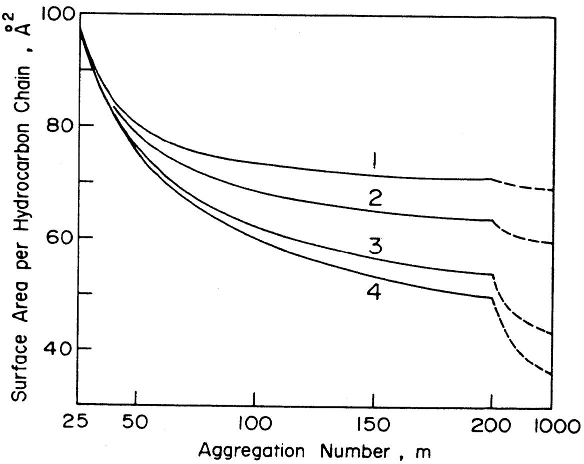

Fig. 6-4. Surface area per hydrocarbon chain as a function of the number of chains per micelle (m'), for the models of Fig. 6-3. Calculations are for $n_c' = 11$, for which equation 6-5 gives $l_{max} = 15.42$ Å, with $b = 0.80\ l_{max}$. An increase in b/l_{max} would make the areas a little smaller and a decrease would make them a little larger. Calculations refer to a distance of 1.5 Å from the smooth interface A in Fig. 6-2, which corresponds to the distance of closest approach of centers of water molecules. Curve 1—prolate ellipsoid, curve 2—cylinder with caps, curve 3—oblate ellipsoid, and curve 4—disk with rounded edge.

This is illustrated by Fig. 6-4. For the same reason the axial asymmetry ratio a/b that is needed for accommodation of a given number of hydrocarbon chains is less for a disklike than for a rodlike micelle. For example, to obtain $m' = 100$ with $n_c' = 11$ and $b = 0.75\ l_{max}$ we require $a/b = 2.2$ for an oblate ellipsoid and $a/b = 5.0$ for a prolate ellipsoid.

Large Micelles, Bilayers and Vesicles

When very large micellar aggregates are formed, there must be a corresponding increase in particle asymmetry. The ellipsoidal models of Fig. 6-3 are then probably no longer realistic because the edges of a very large oblate ellipsoid become very thin and the ends of a large prolate ellipsoid become very pointed. Disks with rounded edges and capped cylinders are probably better geometrical representations for micelles in this category, and some surface area calculations based on these models are given in Table 6-3. For a disk with $b = l_{max}$, A' approaches 21 Å² at the limit of infinite micelle size, which is the cross-sectional area of close-packed hydrocarbon chains in an alkane crystal.

Table 6-3. Surface Areas of Large Micelles for Amphiphiles with One or Two Alkyl Chains[a]

			Area (Å^2) per Head Group	
Model	Hydrocarbon Chains per Particle	Area (Å^2) per Chain	1 Chain per Molecule	2 Chains per Molecule
	$n_C' = 11$, $l_{max} = 15.42$ Å, $h/l_{max} = 0.80$			
Cylinder with caps	100	68.6	68.6	137.2
	1,000	59.8	59.8	119.6
	10,000	58.9	58.9	117.8
	∞	58.8	58.8	117.6
Disk with rounded edge	100	59.9	59.9	119.8
	1,000	36.3	36.3	72.6
	10,000	29.3	29.3	58.6
	∞	26.2	26.2	52.4
(with $h = l_{max}$)	∞	21.0	21.0	42.0
	$n_C' = 16$, $l_{max} = 21.74$ Å, $h/l_{max} = 0.70$			
Cylinder with caps	100	79.9	79.9	159.8
	1,000	67.5	67.5	135.0
	10,000	66.2	66.2	132.4
	∞	66.1	66.1	132.2
Disk with rounded edge	100	72.9	72.9	145.8
	200	59.7	59.7	119.4
	1,000	42.9	42.9	85.8
	4,000	36.4	36.4	72.8
	10,000	34.1	34.1	68.2
	∞	30.1	30.1	60.2
(with $h = l_{max}$)	∞	21.0	21.0	42.0
Spherical vesicle	4,000	31.4	31.4	62.8
	10,000	30.6	30.6	61.2

[a] The figures for $n'_C = 16$ approximate dimensions of natural phospholipids.

Very large disklike micelles are in effect extended sheets, generally called *bilayers*. If the hydrocarbon core is liquid, bilayers will presumably not have perfectly flat surfaces, but will have randomly fluctuating local curvature. As a result of such fluctuations it is possible to eliminate the need for the semitoroidal ring around the edge of a bilayer by formation of *closed vesicles* with an internal solvent-filled cavity. Such vesiculation presents no geometrical problems in a model in which the hydrocarbon is treated as a fluid continuum, but a problem arises when the molecular nature of the associating amphiphile is taken into account because the external surface of a vesicular structure has a larger surface area than the internal surface. This means that the number of molecules in the outward-facing half of the bilayer must be larger than the number of molecules in the inward-facing half. As will be seen in Chapter 11, phospholipids readily form vesicles in which the number of molecules in the outer half of the bilayer has to be almost three times as big as the number in the inner half.

THE PRINCIPLE OF OPPOSING FORCES

The self-association of amphiphiles in aqueous solution into micellar aggregates is accounted for (even predicted) by the hydrophobic effect, but the frequent preference for formation of relatively small micelles, with aggregation number of the order of 100 or less, requires the existence of an opposing force that prevents the growth of the aggregates to larger size. Even in systems where very large micelles are formed (e.g., bilayer sheets), a repulsive force must be invoked to prevent separation of the amphiphile into an entirely separate phase.

It should be noted that the hydrophobic effect per se dictates a lower limit to the micelle size, because a minimum number of amphiphiles have to become associated with each other before an effective elimination of the hydrocarbon–water interface can be achieved. Two or three amphiphiles cannot form a stable micelle, regardless of whether the hydrocarbon core is to be liquid or ordered. Thus micelle formation is necessarily a cooperative process, requiring simultaneous participation by many amphiphilic molecules or ions. The micelles formed cannot have a purely statistical size distribution, but are limited by this factor in how small they can be, in addition to the limitation on large size that may be imposed by the repulsive force.

The repulsive force in micelle formation must come primarily from the head groups. In ionic micelles electrostatic repulsion between like charges is a major factor. In micelles formed by amphiphiles with uncharged head groups a preference for hydration, as opposed to self-association, is involved. Micelles are not formed by simple aliphatic alcohols, which can associate with each other by hydrogen bonds between OH groups, and can thus separate from the aqueous medium as a distinct phase (Fig. 3-1). Amphiphiles with a polyoxyethylene head group, $-(OCH_2CH_2)_xOH$, on the other hand, do form micelles because of the requirement for hydration of the ether oxygen atoms.

The existence of an upper limit to micelle size is now readily visualized in terms of the geometrical requirement that an increase in aggregation number forces amphiphile head groups to approach closer to each other. The repulsive force opposes too close an approach and thereby can keep the micelle size small. For amphiphiles with a single dodecyl chain the optimal aggregation number is frequently near 100, as already noted, and this means (Fig. 6-4) that the optimal separation is reached when the surface area per emerging chain is about 60 to 70 Å^2, corresponding to a separation of about 8 Å. This separation may be considered typical of a wide variety of head groups: amphiphiles with a single alkyl chain can form more tightly packed structures with a smaller head group separation, but usually only under extreme conditions, for example, at very high concentrations as an anhydrous state is approached.

AMPHIPHILES WITH TWO ALKYL CHAINS

The role of head group interactions in the determination of optimal micelle size has an important consequence for amphiphiles containing two alkyl chains attached to a single head group. For a given number of hydrocarbon chains there are now only half as many head groups as there would be for a single chain amphiphile, and the area *per head group* becomes twice as large as the area per emerging chain. Actual figures are given in Table 6-3. Since the repulsive force between head groups should not be significantly affected by whether there are one or two alkyl chains attached to each group, the optimal area per head group for a stable micelle should still be about 60 to 70 Å^2, and the table shows that such a small area can now be attained only by formation of a bilayer sheet or by a closed vesicle bounded by a bilayer. The formation of bilayers or vesicles by biological lipids (Chapter 11) can thus be accounted for by purely geometrical considerations, without the need for new theoretical ideas, and it has been demonstrated (Kunitake and Okahate, 1977) that simple synthetic amphiphiles with two alkyl chains form similar structures.

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SEVEN

THERMODYNAMICS OF MICELLE FORMATION

The thermodynamic principles underlying micelle formation are conceptually simple: the hydrophobic effect provides the driving force for aggregation, whereas repulsion between head groups limits the size that a micelle can attain. However, *both factors* must vary with the micelle size. If, for example, the free energy gained in micellization by virtue of the hydrophobic effect were independent of size, there would be no lower limit to the micelle size and no way to account for the cooperativity with which micelle formation is known to occur. (In fact, as footnote 3 below will show, an optimal micelle size cannot exist unless the hydrophobic contribution to the free energy becomes progressively more favorable even beyond the optimal size.) It is therefore evident that a theoretical treatment that does not explicitly allow for size dependence may account for the occurrence of amphiphile aggregation, but it cannot explain why the aggregation product has the characteristics of micelles instead of being, for example, a statistical aggregate with a broad size distribution, such as is obtained in the formation of condensation polymers.

It is of course easy to see that head group repulsion must increase continuously with micelle size because the geometrical considerations of the previous chapter show that head groups must approach closer to each other as the size increases. The physical basis for the size dependence of the hydrophobic part of the free energy has, however, been recognized only recently (Tanford, 1974a, b), and for this reason all previous treatments of the thermodynamics of micellization, including that in the first edition of *The Hydrophobic Effect*, are inadequate. One previous analysis

(Hall and Pethica, 1967) is thermodynamically exact and includes useful relations for the effect of temperature on micellization, but it lacks explicit expressions for the free energy as a function of size. Two other early reviews (Shinoda et al., 1963; Mukerjee, 1967) present a wealth of experimental data which are discussed within the context of the theoretical relations available at the time.

THERMODYNAMIC FORMULATION

The objective of a thermodynamic treatment is to relate the chemical potential of an amphiphile in the micellar state to the chemical potential of the free amphiphile in solution. Both the inherent part of the chemical potential and the cratic part (p. 6) are affected by micellization, the former because the local environment of the molecule is altered and the latter because aggregation of many particles into a single particle takes place. Since an equilibrium mixture will normally contain micelles with a range of different aggregation states, it is necessary for a rigorous approach to allow explicitly for the size dependence of both contributions to the chemical potential. The simplest procedure is to consider micelles of different size as distinct components of the solution, each characterized by the number m of constituent monomer molecules and by a distinct value of the standard potential, $\mu^{\circ}_{\text{mic},m}$ (per constituent amphiphile molecule). The dependence of the standard potential on the value of m arises from the dependence of surface area on micelle size, as previously discussed in Chapter 6.

The cratic contribution to the free energy *per mol of micelles of size m* is RT ln (mol fraction of micelles of size m), assuming that one is dealing with a solution sufficiently dilute in micelles to permit neglect of nonideality terms arising from interaction of micelles with each other. It is more convenient to express concentrations in terms of mol fraction of monomeric amphiphile incorporated in micelles of size m, which we shall designate by X_m. The cratic contribution per mol of micelles then becomes $RT \ln (X_m/m)$ and the contribution *per mol of amphiphile* is $1/m$ of that, so that the chemical potential of an amphiphile in a micelle of size m becomes

$$\mu_{\text{mic},m} = \mu^{\circ}_{\text{mic},m} + \frac{RT}{m} \ln \frac{X_m}{m} \qquad (7\text{-}1)$$

At equilibrium, $\mu_{\text{mic},m}$ must have the same value for all micellar species and must also be equal to the chemical potential of free amphiphile in solution, as given by equation 2-1. Thus, for any value of m,

$$\mu^\circ_{\text{mic},m} - \mu^\circ_W = RT \ln X_W + RT \ln f_W - \frac{RT}{m} \ln \frac{X_m}{m} \tag{7-2}$$

or, upon rearrangement,

$$\ln X_m = -m \frac{\mu^\circ_{\text{mic},m} - \mu^\circ_W}{RT} + m \ln X_W + m \ln f_W + \ln m \tag{7-3}$$

The overall concentration of amphiphile in micellar form (X_{mic}) and the total concentration in the solution (X_{tot}) are given by

$$X_{\text{mic}} = \sum_{m=2}^{\infty} X_m \tag{7-4}$$

$$X_{\text{tot}} = X_W + \sum_{m=2}^{\infty} X_m \tag{7-5}$$

The lower limit $m = 2$ in the sums of these and subsequent equations is a formal limit: for simple amphiphiles containing a reasonably large hydrocarbon moiety, X_m will not contribute significantly to these sums until m is considerably larger than 2, for reasons already given.

Equation 7-3 is an explicit distribution function for micelle size. In terms of it one can define an optimal size m^* as that value of m for which $\ln X_m$ is maximal at the prevailing monomer concentration X_W, that is,

$$(\partial \ln X_m / \partial m)_{X_W} = 0 \qquad (m = m^*) \tag{7-6}$$

The experimentally measurable number-average or weight-average micelle sizes can also be expressed in terms of equation 7-3. Since the concentration of micelles of size m is X_m/m,

$$\begin{aligned} \bar{m}_n &= \sum_{m=2}^{\infty} X_m \Big/ \sum_{m=2}^{\infty} (X_m/m) \\ \bar{m}_w &= \sum_{m=2}^{\infty} m X_m \Big/ \sum_{m=2}^{\infty} X_m \end{aligned} \tag{7-7}$$

Size distribution functions for many micellar systems are sufficiently narrow so that m^*, $\bar{m}_n$, and $\bar{m}_w$ are nearly the same. The symbol $\bar{m}$ will be used in approximate relations where the distinction is unimportant.

These equations will be used as a basis for the prediction of micellar properties from estimates of the free energy of micellization and, conversely, to obtain information about the free energy of micellization from experimental data. It will be seen that even a crude theory for the calculation of the free energy automatically provides $\mu^\circ_{\text{mic},m} - \mu^\circ_W$ as a function of m, and therefore no simplification of the equations is needed for predictive purposes. However, when the purpose is to analyze experimental data, the size distribution is never available: only an average size ($\bar{m}$) and the total amphiphile in micellar form (equation 7-4) are known. It then becomes necessary to treat all micelles as a single component, in effect assuming that all micelles have the same size $\bar{m}$, and to introduce a standard potential μ°_{mic} (without subscript m) for an amphiphile in any micelle. Equation 7-3 becomes

$$\ln X_{\text{mic}} = -\bar{m}\,\frac{\mu^\circ_{\text{mic}} - \mu^\circ_W}{RT} + \bar{m}\ln X_W + \bar{m}\ln f_W + \ln \bar{m} \tag{7-8}$$

Equation 7-4 becomes unnecessary and $\bar{m}_n = \bar{m}_w = \bar{m}$. It is important to note that μ°_{mic} is not an average of the $\mu^\circ_{\text{mic},m}$ of equation 7-3, but must be more negative than any of them, since X_{mic} must be larger than any individual X_{m}.

In the past micelles have sometimes been considered to be a separate phase from the aqueous solution containing monomeric amphiphile. In that case the Gibbs phase rule would apply, and micelles and free amphiphile could be in equilibrium at a given temperature and pressure only at a single fixed value of X_W, which would effectively be a "solubility." By analogy with equation 2-1, we would have

$$\mu^\circ_{\text{mic}} - \mu^\circ_W = RT\ln X_W + RT\ln f_W \tag{7-9}$$

Phase separation is equivalent to formation of a micelle of infinite size, and equation 7-8 becomes identical to equation 7-9 as $\bar{m}$ approaches ∞.

Alternative Derivation in Terms of Equilibrium Constants

Micelle formation can be expressed in terms of equilibrium constants for the association of monomeric amphiphile (Z) into micelles (Z_m), that is,

$$K_m = [\,Z_m\,]\,/\,[\,Z\,]^m \tag{7-10}$$

where square brackets represent thermodynamic activities. Assuming ideality for the micellar species and replacing its concentration by X_m/m, equation 7-3 is again obtained, with $m(\mu^\circ_{\text{mic},m} - \mu^\circ_W) = -RT\ln K_m$. This method of deriving the equilibrium equations was used by Tanford (1974b) and earlier by Debye (1949). Debye, however, unaccountably omitted the term $m \ln X_w f_w$ when he used equation 7-3 to estimate the optimal micelle size.

CRITICAL MICELLE CONCENTRATION (cmc)

The concept of a "critical concentration" for the formation of micelles from free amphiphile is inexact but convenient. It would be exact if

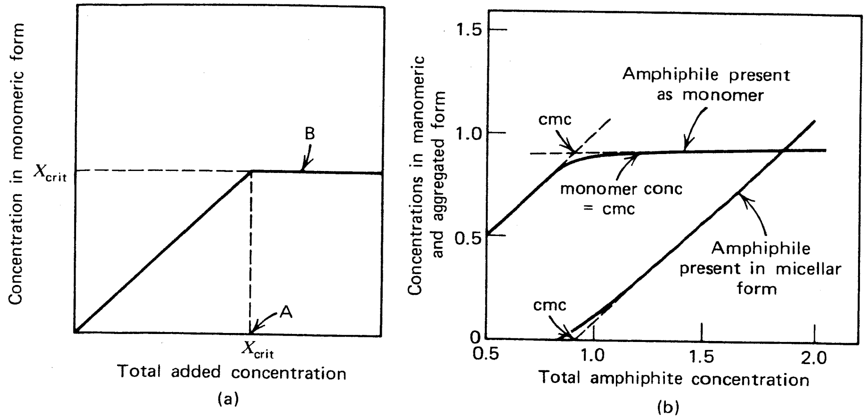

Fig. 7-1. Relation between monomeric concentration in solution and total added concentration in (*a*) true phase separation and (*b*) micelle formation. Figure 7-1*b* is based on equation 7-8 with $\bar{m} = 50$ and $f_w = 1$. Both μ°_{mic} and $\bar{m}$ are assumed independent of concentration within the range covered. The figure shows the concentration of amphiphile present in micellar form as well as the monomer concentration. The dashed lines show empirical procedures for determining the cmc; the point at which the monomer concentration is equal to the cmc is also indicated. Concentration units are arbitrary and could be expressed in grams of amphiphile or in moles based on monomer molecular weight.

micelle formation could be regarded as separation of a distinct phase, and free amphiphile in solution could coexist with the micellar phase at only a single value of X_W (as given by equation 7-9, above). As shown in Fig. 7-1*a*, this unique value of X_W would be measurable in two ways: either as the total added concentration at which phase separation first occurs (point A), or as the free concentration in solution as analytically measured in the presence of the micellar phase (line B).

In fact, micelle formation is not equivalent to phase separation. When micelles and free amphiphiles coexist, X_W is a variable, depending on the concentration of micelles present according to equations 7-3 to 7-5, or equation 7-8 if the approximation that all micelles have the same size is used. However, if $\bar{m}$ is reasonably large ($\bar{m} \gtrsim 50$) X_W changes only slowly as X_{mic} increases, and the transition from the predominantly unassociated amphiphile to the micellar state does in fact occur over a narrow critical range of concentration, approaching true phase separation in sharpness, as illustrated by Fig. 7-1*b*.

It is customary to define a single concentration within this transition zone as a *critical micelle concentration*, generally abbreviated as "cmc." This is usually done by empirical graphical procedures, such as shown by the dashed lines of Fig. 7-1*b*. In practice, X_W and $X_{\rm mic}$ are not necessarily measured explicitly, and the cmc determination is based on a change in slope when an appropriate physical property that distinguishes between micellar and free amphiphile is plotted against total concentration. It is important to emphasize that no procedure can yield a unique critical concentration because none in fact exists, even in terms of explicit measurement of X_W and $X_{\rm mic}$. The lines representing X_W and $X_{\rm mic}$ in Fig. 7-1*b* continue to curve very slightly far from the transition zone, and the cmc value depends to some extent on the concentration range from which the extrapolation to the dashed lines of the figure is performed. However, as long as measurements are derived from data reasonably close to the transition zone, the ambiguity in the cmc generally does not exceed 1 or 2%. The various methods of measurement, and the results obtained by their use, are critically discussed by Mukerjee and Mysels (1971).

An important feature of micelle formation, as previously noted, is that the free amphiphile concentration in equilibrium with micelles changes only slowly with the concentration of micelles. Thus $X_W \simeq$ cmc over a wide range of conditions. In Fig. 7-1*b*, where the empirically established cmc is 0.90 in the arbitrary units that have been used, $X_W = 0.885$ when $X_{\rm mic} = 0.12$, $X_W = 0.90 =$ cmc when $X_{\rm mic} = 0.30$, $X_W = 0.92$ when $X_{\rm mic} = 1.00$, and so on. Though these calculations are based on an assumed constant micelle size, they apply even more forcefully to the real situation of a distribution of micelle sizes because Le Chatelier's principle then requires a gradual increase in $\bar{m}$ with total amphiphile concentration (equation 8-1) and a concomitant sharpening of the transition. An experimentally significant increase in X_W with increasing total amphiphile can occur only when $\bar{m}$ is very small.

At the cmc itself ($X_{\rm tot} =$ cmc), $X_{\rm mic}$ is usually only a small fraction of the total concentration. In the example of Fig. 7-1*b*, at the empirically established cmc, $X_{\rm mic} = 0.035$ cmc. When experimental cmc values are related to $\mu^\circ_{\rm mic} - \mu^\circ_W$ by the approximate equation 7-8, it is convenient to define a parameter $\sigma = X_{\rm mic}/\text{cmc}$, so that $X_W = (1 - \sigma)\,\text{cmc}$. Equation 7-8 can then be written

$$\begin{aligned}(\mu^\circ_{\rm mic} - \mu^\circ_W)/RT = {} & [(\bar{m} - 1)/\bar{m}] \ln \text{cmc} + \ln f_W + \ln (1 - \sigma) \\ & + (1/\bar{m}) \ln (\bar{m}/\sigma)\end{aligned} \tag{7-11}$$

The choice of σ, within limits of 0.01 to 0.10, has only a small effect on calculations made by use of this equation. For very large $\bar{m}$ and with $1 - \sigma$ close to unity, equation 7-11 reduces to equation 7-9 with $X_W =$ cmc, as is to be expected.

It should be noted that it is not possible to eliminate the term $\ln f_W$ from equation 7-11 and other equations of this chapter because of the possibility mentioned earlier (p. 18) that free amphiphile in solution may behave nonideally as a result of interactions not directly related to micelle formation. Such interactions, if significant, cannot be eliminated here by extrapolation to zero solute concentration because one is forced in the study of micelle formation to work at an essentially constant free amphiphile concentration, that is, $X_W \simeq$ cmc.

Experimental cmc Values

Experimental cmc values are available for a large number of amphiphiles (Mukerjee and Mysels, 1971). We shall consider only the simplest amphiphiles, where the hydrophobic moiety is a linear alkyl chain. The cmc values for these compounds exhibit a great deal of regularity.

1. When the amphiphile head group is ionic, the cmc (as well as the average micelle size) is strongly dependent on ionic strength, as shown by Fig. 7-2, and, to a lesser extent, on the specific counterion employed.[1] This presumably reflects a contribution of long-range electrostatic forces to the repulsion between head groups.
2. At high ionic strength, cmc values for ionic amphiphiles are close to the values for amphiphiles with the same alkyl chain, but containing a zwitterionic or highly polar uncharged head group. For example, at 25°C, the cmc is 1.5 *mM* for dodecyl trimethylammonium ion in 0.5 *M* NaBr, 2.4 *mM* for *N*-dodecyl betaine, and 2.0 *mM* for dodecyl dimethylamine oxide. The cmc values are an order of magnitude lower when the head group is only weakly polar, for example, 0.09 *mM* for dodecyl octaethyleneglycol monoether and 0.19 *mM* for dodecyl glucoside.
3. The most prominent regular feature is the dependence of the cmc on the length of the alkyl chain. Both for ionic amphiphiles at constant ionic strength and for all nonionic amphiphiles, the cmc decreases by a factor of 10 when the alkyl chain length is increased by two carbon atoms, as is shown by Fig. 7-3. This corresponds to an increment of about −700 cal/mol per CH_2 group in $\mu^{\circ}_{\text{mic}} - \mu^{\circ}_{W}$.

Experimental measurements of micelle size have been made for relatively few systems, and for some nonionic amphiphiles they are subject to complications to be discussed in the following chapter. For most of the

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[1] Many ionic amphiphiles readily form crystalline precipitates, preventing the formation of micelles below a characteristic temperature, called the *critical micellar temperature*. For Na dodecyl sulfate, in the presence of 0.15 *M* Na^+, this critical temperature is about 23°C (Becker et al., 1975). When divalent counterions are used, the critical temperature is much higher.

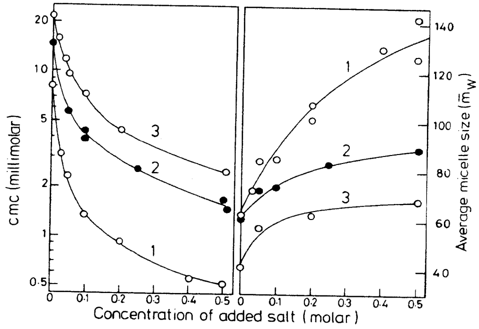


Fig. 7-2. Dependence of the cmc and average micelle size on ionic strength and counterion, at 25°C. Curve 1—Na dodecyl sulfate with added NaCl; curve 2—dodecyl trimethylammonium bromide with added NaBr; curve 3—dodecyl trimethylammonium chloride with added NaCl. Data of Emerson and Holtzer (1967b), Mysels and Princen (1959), Anacker et al. (1964), and Kushner et al. (1957).

data of Fig. 7-3 it is therefore not possible to obtain values of $\mu^\circ_{\text{mic}} - \mu^\circ_W$ by use of equation 7-8. Experimental $\bar{m}$ values are, however, available for the N-alkyl betaines, $RN(CH_3)_2^+CH_2COO^-$ (Swarbrick and Daruwala, 1969, 1970), and for this series of compounds least squares analysis yields

$$\mu^\circ_{\text{mic}} - \mu^\circ_W = 2931 - 732n_C \tag{7-12}$$

where n_C is the number of carbon atoms in the alkyl chain, with an uncertainty of ± 10 cal/mol in the slope and ± 120 cal/mol in the intercept. There being no information on thermodynamic properties below the cmc, $\ln f_W$ in equation 7-8 has been set equal to zero.

It may be noted that if the phase separation equation 7-9 is used, least squares analysis gives

$$\mu^\circ_{\text{mic}} - \mu^\circ_W = 2514 - 709n_C \tag{7-13}$$

which shows that the size-dependent terms of equation 7-8 are relatively unimportant and that reasonably accurate free energy data can be obtained from cmc values even if $\bar{m}$ is not known.

When ionic micelles are studied in the absence of added salt, a much smaller dependence of the cmc on chain length is observed, as is illustrated by the data for alkyl sulfates in Fig. 7-3. The increment $\mu^\circ_{\text{mic}} - \mu^\circ_W$ per added carbon atom is only −418 cal/mol, as compared to the value of about −700 cal/mol applicable to the other data in the figure. Slopes of similar plots for several other ionic amphiphiles, in the absence of added salt, also lie close to −420 cal/mol (Shinoda et al., 1963). These greatly reduced slopes are of course ascribable to the fact that in the absence of

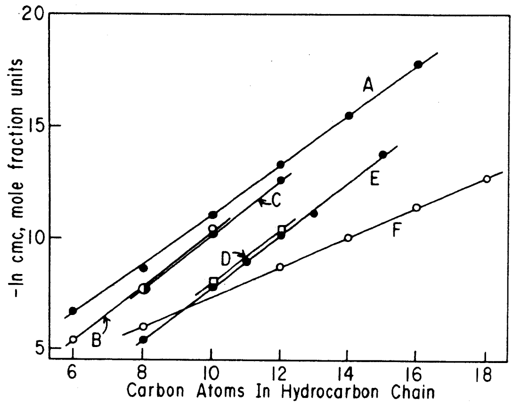


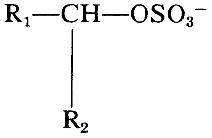
Fig. 7-3. Plots of ln cmc versus hydrocarbon chain length at 25°C, unless otherwise stated. *A*, Alkyl hexaoxyethylene glycol monoethers from Becher (1967). *B*, Alkyl sulfinyl alcohols (Corkill et al., 1966). *C*, Alkyl glucosides from Becher (1967). *D*, Alkyl trimethylammonium bromides in 0.5 *M* NaBr (Emerson and Holtzer, 1967a; Geer et al., 1971). *E*, *N*-Alkyl betaines (Swarbrick and Daruwala, 1969). *F*, Alkyl sulfates in the absence of added salt, at 40°C (Evans, 1956).

added salt, the ionic strength of the solution is determined by the concentration of free amphiphile, that is, it is essentially equal to the cmc when the amphiphile is a 1,1-electrolyte. The ionic strength thus decreases as the alkyl chain is lengthened, partially counteracting the decrease in the cmc that would be expected for a change in n_C at constant ionic strength. A rough calculation was made in the first edition of this book to show that the observed slope is consistent with this explanation.

There exist a large number of cmc measurements of ionic micelles in the absence of added salt, and some interesting observations may be based on them. Klevens (1953), for example, has found that the cmc's of alkyl carboxylates, alkyl ammonium chlorides, and alkyl sulfonates, for the same length of hydrocarbon tail, are nearly the same, but that the alkyl sulfates of a given chain length have cmc's comparable to those of the other amphiphiles with an additional carbon atom. In other words, the extra oxygen atom in the sulfate head group (—O—SO_3^-) behaves as if it were an extra CH_2 group. This result may in part reflect the greater separation between head group charges when the head groups are displaced further from the surface of the micelle core, but it may also indicate that in ionic micelles more than one CH_2 group near the ionic site is incompletely incorporated into the micelle core[2], an effect which would allow substitution of an oxygen atom for a CH_2 group adjacent to the ionic site with little effect on $\mu^\circ_{\mathrm{mic}} - \mu^\circ_W$. The anomalous cmc of dodecyl sulfate persists at high ionic strength, as is evident from a comparison with dodecyl trimethylammonium salts in Fig. 7-2. High ionic strength data for alkyl sulfonates are not available.

The effect of unsaturation on $\mu^\circ_{\mathrm{mic}} - \mu^\circ_W$ has been studied by use of ionic micelles in the absence of added salt, and it appears to be similar to its effect on $\mu^\circ_{HC} - \mu^\circ_W$ as measured by hydrocarbon solubility. Comparison between cmc values for potassium oleate and potassium stearate, and for potassium abietate and potassium dehydroabietate (Klevens, 1953), shows that introduction of a double bond (in these instances near the middle of the hydrocarbon chain) has an effect equivalent to removal of 1 to 1.5 carbon atoms from a saturated fatty acid chain.

An interesting study by Evans (1956) makes it possible to assess the effect of having two separate hydrocarbon chains on an amphiphile molecule with a single head group. Evans measured the cmc of compounds of the type



$$R_1\text{—}\underset{\displaystyle R_2}{\underset{|}{CH}}\text{—}OSO_3^-$$

and some of his results are summarized in Fig. 7-4. The results demonstrate unequivocally that a second hydrocarbon tail, added to an amphiphile molecule already possessing a longer tail, makes a smaller contribution to $\mu^\circ_{\mathrm{mic}} - \mu^\circ_W$ than is made by the longer hydrocarbon chain. On the other hand, the increment per carbon atom added to the longer chain is not affected by the presence of a shorter chain. These results suggest that two hydrocarbon chains on the same amphiphile molecule (in the monomeric state in aqueous solution) tend to associate with each other so that the hydrocarbon–water interface is reduced.

Similar studies with dialkyl dimethylammonium halides (Shinoda et al., 1963, p. 46) lend partial but not complete support for this conclusion. The

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[2] An important theoretical paper by Aniansson (1978) suggests on the basis of the *dynamics* of micelle formation that more than one CH_2 group would protrude at any given instant in about one third of the monomers of a micelle formed by a nonionic amphiphile with a dodecyl alkyl chain. For an ionic amphiphile in the absence of added salt an even greater protrusion is predicted.

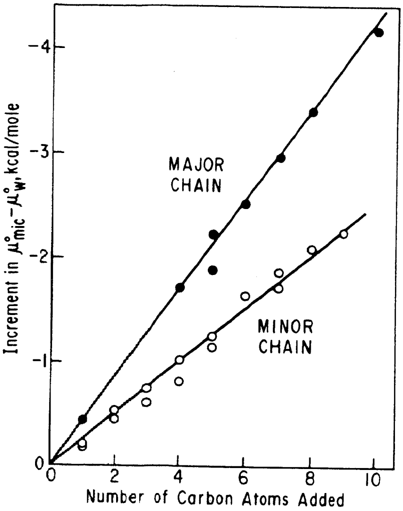

Fig. 7-4. Increment in $\mu^{\circ}_{\text{mic}} - \mu^{\circ}_{W}$ on lengthening the hydrocarbon chains on 1′-(alkyl)′-alkyl-1-sulfates. Data of Evans (1956), at 40°C in the absence of added salt. Major chain data are for single-chain alkyl sulfates with octyl sulfate as reference and for double-chain derivatives with 1-butyl-decyl sulfate and 1-pentyl-hexyl sulfate as reference. Minor chain data represent a variety of compounds, the parent major chain in each case being the reference compound.

cmc of the dioctyl compound in this series is about the same as that of the single-chain undecyl compound. On the other hand, the cmc of the didecyl compound is only slightly higher than that of the compound containing one octyl and one dodecyl chain.

APPLICATION OF THE PRINCIPLE OF OPPOSING FORCES

The principle of opposing forces governing the stabilization of micelles of finite size makes it realistic to divide the free energy of micellization into two factors

$$\mu^{\circ}_{\text{mic},m} - \mu^{\circ}_{W} = U^{\circ}_{m} - \mu^{\circ}_{W} + W_{m} \tag{7-14}$$

$U^{\circ}_{m} - \mu^{\circ}_{W}$, which will be negative in sign, represents the contribution of the hydrophobic effect in favor of association, and W_m, which will be positive, represents the free energy arising from repulsion between head groups. The purpose of this section is to show that both $U^{\circ}_{m} - \mu^{\circ}_{W}$ and W_m can in principle be calculated on the basis of factors already discussed. Not all parameters required for the calculation are known exactly, but approximate estimates can be made to obtain both the magnitudes of $U^{\circ}_{m} - \mu^{\circ}_{W}$ and W_m and their dependence on micelle size. The micelle size distribution function (equation 7-3), the average micelle size, and the cmc can thus all be predicted without the need for any freely adjustable parameters, and the predictions of $\bar{m}_W$ and the cmc for ionic micelles agree with experimental results within entirely reasonable limits.

The surface area per head group will prove to be an important parameter in the calculations. As is illustrated by Fig. 6-4, the area differs for disklike and rodlike micelles, but depends very little on the actual shape within each category. In most situations $\mu^\circ_{\text{mic},m} - \mu^\circ_W$ proves to be more negative (at any given m) for a disklike model than for a rodlike model, which means that a disklike shape is thermodynamically preferred; all calculations made here are therefore based on a disklike model. Situations where rodlike micelles may exist will be discussed in the following chapter.

It will be seen to be appropriate to calculate the area not at the interface between the hydrocarbon core and the hydrophilic mantle of the micelle, but at some distance δx outside it, as in the surface B of Fig. 6-2; the models of Fig. 6-3 are used, with a and b replaced by $a + \delta x$ and $b + \delta x$, respectively. The value of δx required for the calculation of the hydrophobic term (δx_h) will usually not be the same as the value for the repulsive term (δx_r), so that two slightly different areas, A_{hm} and A_{rm}, will be used for each value of m.

The Hydrophobic Component

The free energy gained by removing an alkyl chain from water and placing it in bulk liquid hydrocarbon is about 2100 cal/mol for the terminal CH_3 group and about 820 cal/mol per CH_2 group (p. 15). When the transfer is for a free hydrocarbon molecule from water to the inside of a micelle, the figure per CH_2 group is less (770 cal/mol; equation 6-2) because of the restricted freedom of motion inside a micelle. The figure for an alkyl chain of a micelle-forming amphiphile should be even lower because one end of the chain is now anchored to the amphiphile head group in the micellar mantle. This further decrement can actually be obtained experimentally because, when all terms contributing to the free energy of micellization have been evaluated, it can be demonstrated that $U^\circ_m - \mu^\circ_W$ is the only term contributing significantly to the dependence of the cmc of a homologous series of amphiphiles on the length of the alkyl chain. The CH_2 group contribution to $U^\circ_m - \mu^\circ_W$ can thus be obtained from $RT\ \partial \ln \text{cmc}/\partial n_C$, and Fig. 7-3 then gives a value of −700 cal/mol. The methyl group at the end of an alkyl chain is at the center of the micellar core and should be as free to move, relative to the rest of the chain, as in bulk liquid hydrocarbon, and its contribution to $U^\circ_m - \mu^\circ_W$ should therefore remain at the value for transfer to bulk hydrocarbon, that is, − 2100 cal/mol. It will be assumed that the CH_2 group proximal to the amphiphile head group does not enter the micelle core (p. 52), so that the sum of contributions for complete removal of an alkyl chain of n_C carbon atoms from water is $-[2100 + (n_C - 2)\,700]$ cal/mol.

There is evidence from spectroscopic relaxation experiments that several CH_2 groups within the hydrocarbon core of a micellar structure (those nearer the head group, cf. p. 49) have less freedom of motion than CH_2 groups nearer the center of the core. Since the smallest value of n_C for the data of Fig. 7-3 is 6, the CH_2 group contribution of -700 cal/mol applies to CH_2 groups added beyond the restricted region, and those nearer the surface might make a somewhat smaller contribution. In addition, the data of Klevens cited on p. 68 (see also footnote 2 on p. 69) suggest the possibility that more than one CH_2 group near an amphiphile head group might be incompletely incorporated within the micelle core. Both these factors would make the free energy for removal of the alkyl chain from water less negative than the figure given in the last paragraph. It is appropriate therefore to add a positive constant, C, which cannot be evaluated. It should be independent of n_C and can reasonably be expected to be numerically about equal to or less than the contribution of one CH_2 group, that is, $C \leqslant +700$ cal/mol.

The preceding calculations apply if the hydrocarbon incorporated into the core of a micelle is completely shielded from contact with water, but shielding in a small micelle has to be incomplete because the core/water interfacial area is typically about 60 Å^2 per emerging alkyl chain (p. 55), whereas the area physically occupied by the atoms of the chain itself is only about 21 Å^2, which is the cross-sectional area in the crystalline state and also the limiting area per chain in a compressed amphiphile monolayer. There has to be contact between hydrocarbon and water at the unoccupied part of the area, and this requires a correction to the calculation based on complete shielding, which will depend on the actual area A_{hm} per emerging chain, which in turn depends on m (Fig. 6-4). The correction term can be obtained in free energy units by using the figure of 25 cal/mol per Å^2 of hydrocarbon–water contact area given on p. 8. This figure was based on contact areas calculated at the distance of closest approach of water molecules to the hydrocarbon surface, which is 1.5 Å, so that δx_h as defined above must also be set equal to 1.5 Å. To account for surface roughness (Fig. 6-2) A_{hm} has to be larger than one would estimate for the smooth surface of a geometrical model, by an extent which depends on the surface curvature. One way to allow for this is to use an artificially large value of δx_h, and some simple calculations suggest that $\delta x_h \sim 3.0$ to 3.25 Å (which gives an area increase of 15 to 20% for a small micelle) would be appropriate. The final expression for $U_m^\circ - \mu_W^\circ$ (in cal/mol with A_{hm} in Å^2) then becomes

$$U_m^\circ - \mu_W^\circ = -2100 - (n_C - 2)\,700 + 25\,(A_{hm} - 21) + C \quad (7\text{-}15)$$

The only term in this equation that depends on m, and therefore the only term affecting the calculation of micelle size, is the term in A_{hm}. As Fig. 6-4 shows, A_{hm} decreases continuously with micelle size, causing $U_m^\circ - \mu_W^\circ$ to become more negative.[3]

The freedom of motion of hydrocarbon chains, especially near the head group, may also depend on the value of m since the packing of chains becomes more efficient (at least for disklike micelles) as the micelle size increases. In terms of equation 7-14 this would mean that the parameter C would increase slightly with increasing m, that is, the effect on $U_m^\circ - \mu_W^\circ$ would be in the opposite direction from the effect of the change in area, and in the same direction as the effect on W_m. In view of the present uncertainty in the calculation of W_m and its dependence on size (see below), possible thermodynamic effects of a dependence of C on m are within the overall uncertainty of the calculations.

The Repulsive Component

An a priori calculation of W_m is more difficult. For nonionic amphiphiles, where the repulsion is presumably primarily steric in origin, no explicit functions have been proposed. In ionic micelles, the electrostatic charge on the head group can be expected to be a major factor, and a naive way to evaluate W_m might be to equate it with the work that has to be done to place charges on a sphere of suitable radius where the area per charge is the same as the actual area A_{rm} on the micelle surface. Within the approximation that this involves, the Debye-Hückel theory can be used for the calculation. The usual expression in terms of the total number of charges (Tanford, 1961, equation 26-30) is readily converted to W_m per head group (instead of for the whole sphere) in terms of the area per head group, giving

$$W_m = \frac{2\pi\epsilon^2 r_0 (1 + \kappa a_i)}{DA_{rm}\,(1 + \kappa r_0 + \kappa a_i)} \quad (7\text{-}16)$$

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[3] It is easy to see that a negative value for $d(U_m^\circ - \mu_W^\circ)/dm$ is essential if a micelle size distribution function with an optimal size is to be obtained. Combining equations 7-6 and 7-2 we see that

$$\partial\,(\mu_{\text{mic},m}^\circ - \mu_W^\circ)\,/\,\partial m = (RT/m^2)\,[1 + \ln (X_m/m)]$$

when $m = m^*$. Since $X_m << 1$, the right-hand side of this equation must be negative. Since dW_m/dm is necessarily positive, $d(U_m^\circ - \mu_W^\circ)/dm$ has to be negative.

where r_0 is the radius of the sphere, a_i is the average radius of mobile ions in the surrounding electrolyte, ϵ is the electronic charge, D is the dielectric constant of the medium, and κ is the usual Debye-Hückel reciprocal length, proportional to the square root of the ionic strength. Equation 7-16 is in molecular units and must be multiplied by Avogadro's number and converted to cal/mol for use with equation 7-15.

This simple approach is unfortunately not valid because the high charge density generated by the closely spaced head groups leads to substantial charge neutralization by counterions, even in the absence of added salt (e.g., Sasaki et al., 1975; Bunton et al., 1977). It would also be invalid to base the calculation on the residual unneutralized charge because binding of counterions involves an increase in cratic free energy, due to removal of the counterions from the solution (Mukerjee, 1969), and this is by no means insignificant in comparison with W_m as calculated by equation 7-16. It should be recalled (p. 66) that cmc values for ionic amphiphiles at high ionic strength, where the charges must be almost completely neutralized, are still an order of magnitude larger than cmc values for amphiphiles with a similar alkyl chain and a weakly polar nonionic head group, indicating that W_m is still much larger than when steric factors alone contribute to it.

An alternative approach is to abandon the attempt to calculate W_m and to base it instead on experimental data. As Chapter 10 will show, the work of compression of surface monolayers can in principle be used as a measure of intermolecular forces. For an amphiphile monolayer adsorbed at a hydrocarbon–water interface, the alkyl chains will be in the hydrocarbon medium and should as a first approximation mix ideally with the solvent. The work of compression in excess of the ideal work should therefore arise largely from repulsion between head groups on the aqueous side of the interface. One can then make the assumption that the excess work at a given area per molecule obtained from the compression curve can be equated with W_m for the same area at the surface of a micelle. The monolayer experiments are subject to considerable sources of error, but reasonably accurate data are available for $—N(CH_3)_3^+$ and for $—OSO_3^-$ in 0.1 M NaCl. Expressions for W_m based on these results (see Chapter 10 for method of calculation) are

$$W_m = 1.51 \times 10^5/A_{rm} - 8.3 \times 10^4/A_{rm}^2 - 2.4 \times 10^7/A_{rm}^3 \quad (7\text{-}17)$$

$$W_m = 1.07 \times 10^5/A_{rm} + 5.4 \times 10^5/A_{rm}^2 - 3.6 \times 10^7/A_{rm}^3 \quad (7\text{-}18)$$

equation 7-17 being for $—N(CH_3)_3^+$ and equation 7-18 for $—OSO_3^-$. It is not possible to obtain comparable data for nonionic amphiphiles because of their high solubility in a hydrocarbon medium.

The location of charged groups for the calculation of A_{rm} is determined by molecular dimensions, with allowance for the presence of one CH_2 group of the alkyl chain outside the hydrophobic core of the micelle. The appropriate values of δx_r are 2.5 to 3 Å and 4 to 4.5 Å, respectively, for the centers of charge of $—CH_2N(CH_3)_3^+$ and $—CH_2OSO_3^-$, including allowance for the fact that surface roughness will increase the distance from the time-averaged surface of the hydrophobic core (Fig. 6-2).

Calculated Results

Numerical calculations based on equations for $U_m^\circ - \mu_W^\circ$ and for W_m require the calculation of $\ln X_m$ as a function of m at a series of values of X_W (equation 7-3), which together with equations 7-4 and 7-5 yield all micellar properties as a function of the total amphiphile concentration. The cmc is obtained as that value of X_{tot} at which the ratio X_{mic}/X_W attains an appropriate value, generally ~ 0.05. Shortcuts to shorten the time of calculation have been described (Tanford, 1974b).

Table 7-1 summarizes results of calculations for disklike ionic micelles,

Table 7-1. Calculated Properties for Ionic Micelles at 20°C[a]

Amphiphile	Ionic Strength	m^*	cmc(mol/liter)
Using Equation 7-16 for W_m			
$C_{12}H_{25}OSO_3^-$	0.02	26	0.016
	0.05	32	0.0074
	0.10	39	0.0042
	0.20	48	0.0024
	0.50	64	0.0013
Using Equation 7-17 or 7-18 for W_m			
$C_{12}H_{25}N(CH_3)_3^+$	0.10	60	0.0062
$C_{12}H_{25}OSO_3^-$	0.10	93	0.0018
Experimental Values[b]			
$C_{12}H_{25}N(CH_3)_3^+$	0.10	59	0.0066
$C_{12}H_{25}OSO_3^-$	0.10	95	0.0015

[a] With $\delta x_h = 3.25$ Å, $\delta x_r = 2.5$ Å for $C_{12}H_{25}N(CH_3)_3^+$ and $\delta x_r = 4.0$ Å for $C_{12}H_{25}OSO_3^-$. The adjustable constant C was set equal to zero when equation 7–16 was used and to +500 cal/mol when equation 7-17 or 7-18 was used.

[b] From Emerson and Holtzer (1967b). The figure for micelle size is the weight-average association number, $\bar{m}_w$, which in these systems is experimentally indistinguishable from m^* (see text).

obtained by using the expressions for W_m for ionic head groups given above. It is seen that even use of the unrealistic equation 7-16 for W_m does not lead to completely absurd results. Virtually perfect agreement with experiment is obtained when equations 7-17 and 7-18 are used. The value of the adjustable parameter C that is required to obtain agreement between calculated and experimental cmc values is 500 cal/mol, that is, about 80 cal/mol per "restricted" CH_2 group. The model used for evaluation of A_{hm} and A_{rm} was the disk with a semitoroidal ring around the edge (Fig. 6-3*b*); virtually identical results were obtained when an oblate ellipsoid model was used.

Figure 7-5 shows the size distribution functions corresponding to the calculations using equations 7-17 and 7-18. The size distributions are seen to be quite narrow, and this is nearly always true for disklike micelles when m^* is small, even if quite different expressions for W_m are used (e.g., expressions that might be applicable to nonionic amphiphiles), because the head group area always changes fairly steeply with m for small disklike micelles, as exemplified by Fig. 6-4. One consequence of this is that $m^* \simeq \bar{m}_n \simeq \bar{m}_w$, as previously noted. Another is that the parameter $\mu^\circ_{\text{mic}} - \mu^\circ_W$ which one obtains from experimental data by equation 7-8 when the size distribution is not known is not greatly different from the value of $\mu^\circ_{\text{mic},m} - \mu^\circ_W$ for $m = m^*$. For the distribution shown in Fig. 7-5 for sodium dodecyl sulfate, for example, $\mu^\circ_{\text{mic},m} - \mu^\circ_W$ for $m = m^*$ is -5926 cal/mol and $\mu^\circ_{\text{mic}} - \mu^\circ_W$ is -5948 cal/mol.

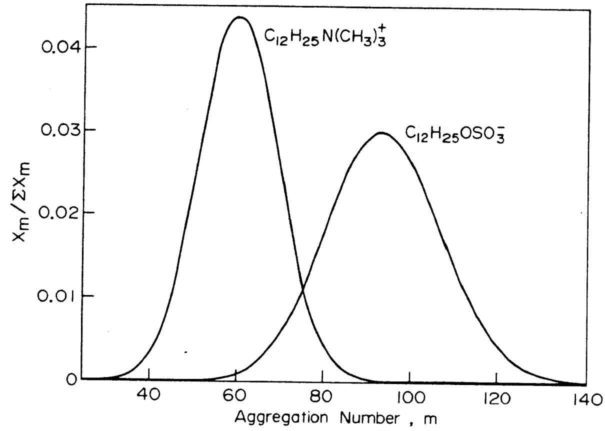

Fig. 7-5. Size distribution functions for micelles formed by $C_{12}H_{25}N(CH_3)_3^+$ and $C_{12}H_{25}OSO_3^-$, using equations 7-17 and 7-18 for W_m.

As has been pointed out, the calculations made here were for a disklike model of the micelle, but the equations do not actually require limitation to one kind of model. To be rigorous, it would be appropriate to make two calculations for each value of m when equation 7-3 is used, one for a disklike micelle and one for a rodlike micelle, and to include both values of X_m in the sums of equations 7-4 and 7-5. When this is done for the parameters used in the present calculation, the concentrations of the rodlike species turn out to be negligible. This is probably a general result, applicable to all systems in which relatively small micelles are formed, provided that one is dealing with reasonably dilute solutions.

EFFECT OF TEMPERATURE

The effect of temperature on the cmc of ionic or zwitterionic micelles is small: $\bar{H}^\circ_{\text{mic}} - \bar{H}^\circ_W$ is close to zero at 25°C for all micelles that have been investigated (e.g., Goddard and Benson, 1957; Emerson and Holtzer, 1967b; Swarbrick and Daruwala, 1969; Shinoda et al., 1963). For nonionic micelles the enthalpy of micellization is positive (e.g., Corkill et al., 1964 a, b; Corkill et al., 1966; Muller and Platko, 1971). In all cases therefore the driving force for micelle formation is a positive entropy change, as is to be expected for a phenomenon that is a manifestation of the hydrophobic effect. The enthalpy change is temperature dependent in all cases: values of $(\bar{C}^\circ_p)_{\text{mic}} - (\bar{C}^\circ_p)_W$ between -50 and -100 cal/deg-mole have been reported for all amphiphiles investigated, which is less than expected from the hydrophobic component of the free energy alone, considering the length of the alkyl chains (Table 4-1). For ionic micelles a positive contribution to ΔC_p is expected as a result of counterion binding to head groups (p. 27). The available data are not sufficiently complete or precise to merit a quantitative discussion.

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EIGHT

MICELLE SIZE AND SHAPE

Molecular weights of micelles and hydrodynamic properties reflecting their shape can be measured by the same methods that are used for true macromolecules. Most of the existing molecular weight information has been obtained by use of light scattering. Only data for amphiphiles with single linear saturated alkyl chains will be considered here, amphiphiles with weakly polar head groups being represented by the alkyl polyoxyethylene ethers, $R(OCH_2CH_2)_x$ OH, which will be abbreviated as $R(OE)_x$ OH.

The molecular weight data for these micelles show much greater diversity than the cmc values discussed in Chapter 7. Table 8-1, for example, shows the weight average aggregation numbers of a series of substituted ammonium salts, all with a decyl hydrocarbon chain, and all having comparable cmc values. The values of $\bar{m}_w$ are evidently greatly influenced by the substitution of CH_3 groups for H atoms on the ionic head group. The difference between the first two entries in the table suggests that the change in counterion from Cl^- to Br^- may also have an effect, but the total salt concentration is also different in the two experiments; as Fig. 8-1 shows, the concentration of salt has an enormous effect on $C_{12}H_{25}NH_3^+$, but the effect on $C_{12}H_{25}N(CH_3)_3^+$ is much less. Electrolyte concentration has very little influence on micelles formed by zwitterionic or nonionic amphiphiles, and results quoted for amphiphiles in this category have mostly been obtained in the absence of added salt.

Figure 8-2 shows the effect of changing alkyl chain length. In terms of the theoretical equations of the previous chapter, the W_m function should be independent of chain length and $U_m^\circ - \mu_W^\circ$ should be altered only by the change in the numerical value of n_C. When calculations are made on this

Table 8-1. Micelle Aggregation Numbers for Decyl Ammonium Salts[a]

	ln cmc (mol fraction units)	$\overline{m}_w$
$C_{10}H_{21}NH_3^+Cl^-$	−7.40	78
$C_{10}H_{21}NH_3^+Br^-$	−8.15	1100
$C_{10}H_{21}NH_2(CH_3)^+Br^-$	−7.93	670[b]
$C_{10}H_{21}NH(CH_3)_2^+Br^-$	−8.44	69
$C_{10}H_{21}N(CH_3)_3^+Br^-$	−8.03	48

[a] From Geer et al. (1971). For decylammonium chloride the solvent was 0.1 *m* NaCl, 30°C; for all other data it was 0.5 *m* NaBr, 25°C. Molecular weights were determined by light scattering.

[b] Light-scattering plots were curved, and a value of $\overline{m}_w = 270$ and a somewhat smaller cmc would have been obtained from data at lower amphiphile concentration.

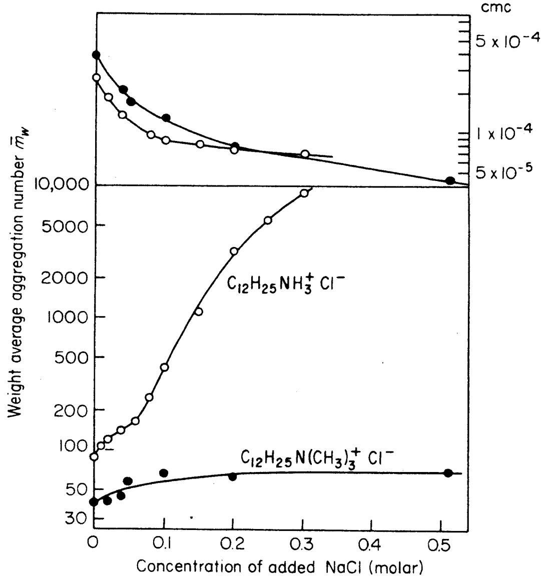

Fig. 8-1. Effect of added electrolyte on micelle aggregation numbers of two structurally similar ionoc amphiphiles. The corresponding cmc values (mol fraction units) are shown at the top of the figure. Data for the ammonium salts are for 30°C (Kushner et al., 1957), and those for the trimethyl ammonium salt are for 23° to 25°C (Emerson and Holtzer, 1967; Kushner et al., 1957).

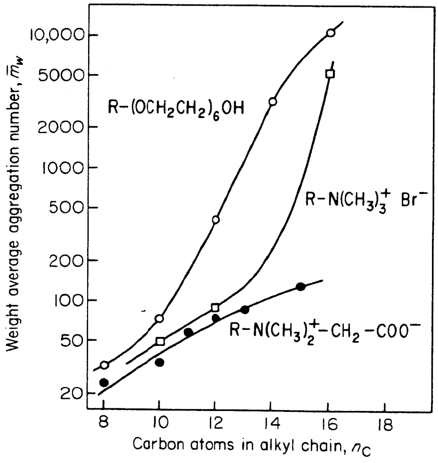

Fig. 8-2. Weight average micelle aggregation numbers, determined by light scattering, as a function of hydrocarbon chain length. The data for the alkyl hexaoxyethylene glycol monoethers are from Balmbra et al. (1964), and those for *N*-alkyl betaines are from Swarbrick and Daruwala (1970). The solvent in both cases was H_2O. The data for the alkyl trimethyl ammonium salts were obtained in 0.23 to 0.50 *M* NaBr and are taken from Geer et al. (1971), Emerson and Holtzer (1967), and Anacker (1970). All data refer to 25°C.

basis for the ionic amphiphiles considered in the last chapter, the main influence of a change in alkyl chain length is predicted to be on the cmc, and only a moderate increase in $\bar{m}_w$ with n_C is expected (Tanford, 1974), such as is shown in the figure for the *N*-alkyl betaines.[1]

A unique feature of the data for the nonionic amphiphiles $R(OE)_x OH$ is a huge dependence on temperature. For example, the large effect of n_C on $\bar{m}_w$ for $R(OE)_6 OH$ at 25°C shown in Fig. 8-2 is not seen in similar data for the same compound at 4°C (Corkill and Walker, 1972). At that temperature the chain length dependence (to $n_C = 14$) is close to that shown in Fig. 8-2 for *N*-alkyl betaines. Results for $C_{12}H_{25}(OE)_6 OH$ over a range of temperatures are shown in Fig. 8-3. Since ΔH for the hydrophobic effect is very small (Chapter 4) one would expect little effect of temperature on micellar properties unless there is a large effect of temperature on head group interactions; very little dependence of size on temperature is observed for the *N*-alkyl betaines where the repulsion between head groups is presumably electrostatic in origin.[1]

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[1] Both the cmc and the $\bar{m}_w$ values for *N*-alkyl betaines are virtually identical to the same parameters for alkyl trimethylammonium bromides in 0.5 *M* NaBr. (The experimental point for the latter at $n_C = 16$ is for 0.23 *M* NaBr). This suggests that the repulsive force between the betaine head groups in water is similar in magnitude to the repulsive force between the ionic head groups of $RN(CH_3)_3^+$ at high ionic strength, and this is not unreasonable since the $-N(CH_3)_2^+-$group of the betaines must lie at the very surface of the hydrophobic core of the micelles, whereas the terminal COO^- group can extend into the surrounding solvent with some freedom of motion, that is, it will resemble the diffuse layer of Br^- counterions surrounding $RN(CH_3)_3^+$ micelles at high ionic strength.

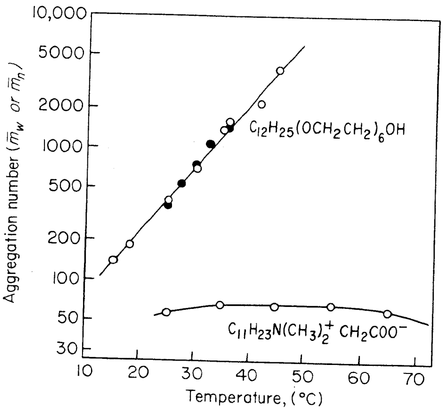

Fig. 8-3. Micelle aggregation number as a function of temperature. Data for *N*-undecyl betaine from Swarbrick and Daruwala (1970); data for dodecyl hexaoxyethylene glycol monoether from Balmbra et al. (1962) and Attwood et al. (1970). Open circles represent $\bar{m}_w$ by light scattering, and filled circles represent $\bar{m}_n$ by osmotic pressure.

MICELLE SIZE DISTRIBUTION

The dependence of molecular weight on concentration is quite generally a direct manifestation of size distribution. For micelles it is readily shown from the definitions of size averages in equations 7-7 (e.g., Hall and Pethica, 1967) that

$$\begin{aligned} d\bar{m}_n/d \ln X_{\text{mic}} &= \bar{m}_n(\bar{m}_w - \bar{m}_n)/\bar{m}_w \\ d\bar{m}_w/d \ln X_{\text{mic}} &= \bar{m}_z - \bar{m}_w \end{aligned} \tag{8-1}$$

where $\bar{m}_z$ is $\Sigma m^2 X_m / \Sigma m X_m$. The differences between the various averages, such as $\bar{m}_w - \bar{m}_n$, are of course a measure of the breadth of the size distribution and the differences become very small when the size distribution is narrow and symmetrical, as in Fig. 7-5.

When large micelles are formed, the observed size usually depends strongly on the amphiphile concentration, as is illustrated by Fig. 8-4, and careful extrapolation to the cmc usually leads to $\bar{m}_w$ values that are not remarkably higher than those observed for amphiphiles of similar alkyl chain length which maintain small size under all conditions. This behavior indicates that large micelles always have a very broad distribution function for micelle size. When the steepness of the concentration dependence is as extreme as in Fig. 8-4, it suggests in fact the existence of a *bimodal*

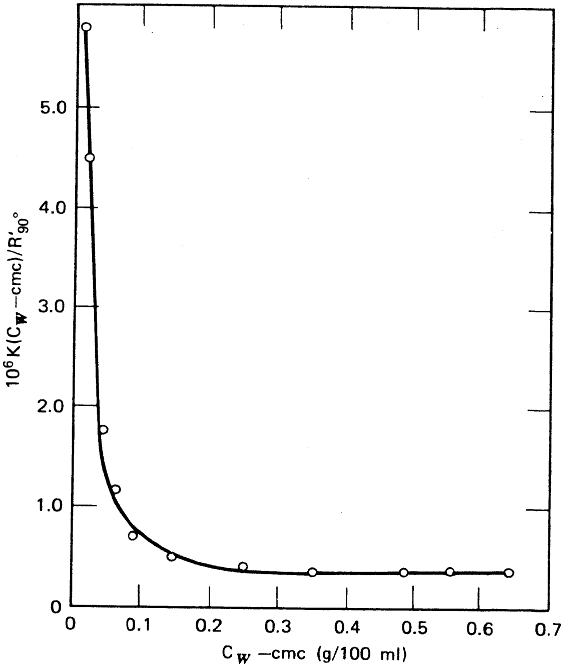

Fig. 8-4. Debye light-scattering plot for $C_{16}H_{33}(OCH_2CH_2)_7OH$ at 25°C taken from Attwood (1968). Extrapolation of the points at highest concentration to zero micelle concentration leads to $m_w = 2600$. Extrapolation of the steeply rising portion of the curve leads to $\bar{m}_w = 145$.

distribution, that is, a distribution function that may be similar to that of Fig. 7-5 at small values of m, but contains a second population at much higher m values, which presumably would have to reflect the existence of micellar aggregates that are fundamentally different from the micelles with small values of m. As shown by a general analysis of molecular size averages and distributions (Tanford, 1961; Section 8), such a bimodal distribution can lead to very large values for $\bar{m}_w - \bar{m}_n$, $\bar{m}_z - \bar{m}_w$, and so on.

It may be noted in this connection that there is an inconsistency in the equivalence of $\bar{m}_w$ and $\bar{m}_n$ values for $C_{12}H_{25}(OE)_6$ OH shown in Fig. 8-3. This is one of the systems with a pronounced dependence of molecular weight on concentration, and a concomitant requirement for a large difference between $\bar{m}_w$ and $\bar{m}_n$.

MICELLE SHAPE

Conclusions about micellar shape may be based on the diffusion coefficient, intrinsic viscosity, or light scattering asymmetry. When the hydrodynamic properties are used, it is necessary to analyze the results in terms of equations for oblate and prolate ellipsoids, because equations for other possible disklike or rodlike shapes do not exist. Ellipsoid dimensions are obtained by calculating the dimensions of the hydrocarbon core of the micelle for m equal to the observed $\bar{m}_w$, as already described (p. 54), and then adding to it a value for the thickness of the head group mantle (δr) based on geometrical considerations. All solvent trapped in the head group mantle is assumed to be an inherent part of the hydrodynamic particle. In polyoxyethylene derivatives, the oxyethylene chains are randomly coiled and extend quite far from the core surface (Tanford et al., 1977), and δr values tend to be at least as large as the minor axis (b in Fig. 6-3) of the micelle core.

Some results obtained from viscosity measurements are shown in Table 8-2. The first two entries are for small micelles, one formed by an ionic and one by a nonionic amphiphile. The data demonstrate unambiguously that these micelles have a shape closer to an oblate than to a prolate ellipsoid and thus support the preference for a disklike shape deduced from thermodynamic and geometrical considerations at the end of the last chapter. When very large micelles are formed, on the other hand, they appear to be sometimes disklike and sometimes rodlike. The results for $C_{12}H_{25}NH_3^+ Cl^-$ clearly indicate a disklike shape, but the viscosity data for $C_{16}H_{33}(EO)_7OH$, while less than predicted for a rigid prolate ellipsoid, are much too large for a disklike shape and have to be interpreted in terms of some kind of extended rod. Light-scattering measurements (Debye and Anacker, 1951; Anacker, 1970) indicate that the large micelles formed by $C_{16}H_{33}N(CH_3)_3^+ Br^-$ in 0.23 M NaBr (Fig. 8-2) are also rod shaped, and there is evidence (e.g., Mazer et al., 1976; Corti and Degiorgio, 1979) that sodium dodecyl sulfate, at ionic strength above 0.5 and at high amphiphile concentration, may form moderately large rod-shaped micelles, presumably present in equilibrium with the disk-shaped micelles that predominate at lower ionic strength.

MECHANISM OF FORMATION OF VERY LARGE MICELLES

The foregoing data show that small changes in an amphiphile head group, which leave the cmc virtually unchanged, can greatly affect the micelle size and shape. This result is not inconsistent with the thermodynamic

Table 8-2. Intrinsic Viscosity and Micelle Shape

	Micelle Size[a] ($m = \bar{m}_w$)	Thickness of Head Group Layer (Å)	[η] in cc/g		
			Calculated		
			Prolate	Oblate	Observed
$C_{12}H_{25}OSO_3Na$[b]	100	4	6.0	3.4	3.3
$C_{12}H_{25}(EO)_8OH$[c]	120	14	7.5	4.9	4.2
$C_{12}H_{25}OSO_3Na$[d]	540	4	49.3	4.3	13.9
$C_{12}H_{25}NH_3Cl$[e]	172	3	12.4	4.1	3.4
	260	3	20.9	4.4	3.8
	430	3	42.6	4.9	4.4
	1170	3	218	6.6	6.0
	3300	3	1340	9.6	15
$C_{16}H_{33}(EO)_7OH$[f]	2600	12	360	5.5	107
	5530	12	1330	6.9	176
	8840	12	3060	8.1	260

[a] For large micelles with broad size distributions the "average micelle" seen by viscosity measurements is not the same as the micelle with $m = \bar{m}_w$. For rod-shaped micelles, the observed value of [η] should be larger than that calculated for $m = \bar{m}_w$; for disklike micelles it should be smaller.

[b] Based on measurements at 25°C in the range of ionic strength 0.1 to 0.2 from several laboratories. Measurements of diffusion coefficients support a disklike shape.

[c] Tanford et al. (1977). The frictional coefficient calculated from the sedimentation velocity supports a disklike shape.

[d] Data for *high ionic strength* (0.6 *M* NaCl) at 25°C and at an amphiphile concentration of 20 mg/cc (Corti and Degiorgio, 1979). The experimental [η] value is calculated from the diffusion coefficient measured by these workers.

[e] Data of Kushner et al. (1957), with generation of different micelle sizes by changing the ionic strength (Fig. 8-1). The same data have been interpreted by Stigter (1966) in terms of a flexible rodlike micelle.

[f] Data of Attwood (1968), with generation of different micelle sizes by changing the temperature (Fig. 8-3).

theory outlined in the previous chapter, since head group interactions affect the cmc through the *magnitude* of W_m, whereas it is the *derivative* dW_m/dm that determines the size distribution and m^*. It requires only moderate flattening of the curve of W_m versus m to produce a broad size distribution and a concomitant increase in micelle size as the concentration rises above the cmc. This mechanism may apply to the formation of large micelles by $C_{12}H_{25}NH_3^+\ Cl^-$, since the viscosity data indicate that the large micelles retain a disklike shape and a bimodal distribution thus appears to be excluded. The derivative dW_m/dm may be replaced by the product $(dW_m/dA_{rm})(dA_{rm}/dm)$, and, since the area per head group is a relatively steep function of m for disklike micelles, a diminution in dW_m/dA_{rm} would be required. It is not hard to imagine why dW_m/dA_{rm} might be much less for RNH_3^+ than for $RN(CH_3)_3^+$ because counterions can approach much closer to the center of positive charge in $—NH_3^+$ than in $—N(CH_3)_3^+$. They can therefore be bound more tightly and might tend to be shared between neighboring head groups, thereby largely nullifying the increase in W_m as head groups approach closer to each other.

It is not easy to visualize what factors might favor the formation of rodlike micelles. One difficulty is the problem posed by the need to pack hydrocarbon chains into a space that becomes increasingly constricted as the center of the rod is approached. A second difficulty is that surface areas are much higher for rodlike micelles (Fig. 6-4); this might favor rodlike micelles when the head group repulsion factor W_m is exceptionally large, but the systems for which the hydrodynamic properties of Table 8-2 suggest a rodlike shape are in fact those for which W_m is exceptionally small, that is, one of the nonionic amphiphiles and sodium dodecyl sulfate at *high* ionic strength. (See the following section for ionic amphiphiles in the absence of added salt.)

It is important to note, therefore, that the observation of a rodlike particle by hydrodynamic measurements does not necessarily imply that the particle is a single micelle, formed by elongation of a small micelle, that is, by an increase in the ratio a/b of Fig. 6-3 without change in the basic arrangement of the amphiphile molecules. It is also possible to form rodlike hydrodynamic particles by secondary aggregation of small micelles into rod-shaped stacks, leaving the size and shape of the individual primary micelles unaltered or only minimally altered. Evidence suggesting that the large micelles formed by polyoxyethylene derivatives and by Na dodecyl sulfate at high salt concentrations are in fact secondary aggregates of this kind is provided by the quite uncharacteristic sensitivity of the phenomenon to temperature, as shown, for example, for $C_{12}H_{25}(OE)_6OH$ in Fig. 8-3. The micelle size and asymmetry increase dramatically with increasing temperature. If the temperature is increased beyond the range covered by the figure or by the data for $C_{16}H_{33}(OE)_7OH$ in Table 8-2, the solutions become cloudy as a result of separation of a highly aggregated amphiphile phase, and the temperature dependence of $\bar{m}_w$ seen while the solutions are still clear is reasonably attributed to formation of aggregates of intermediate size. Ionic amphiphiles do not exhibit the "cloud point" phenomenon, but they form a hydrated crystalline phase as the temperature is reduced. The formation of large asymmetric micelles by Na dodecyl sulfate in 0.6 *M* NaCl has been shown to be favored by a *decrease* in temperature, suggesting that the large micelles are "embryos" of the crystalline phase (Corti and Degiorgio, 1979).

Other evidence is provided by the sensitivity of the formation of large micelles by polyoxyethylene derivatives to the length of the polyoxyethylene chain. Thus, at 25°C, there is an abrupt all-or-none difference between $C_{12}H_{25}(OE)_6OH$ and $C_{12}H_{25}(OE)_8OH$ (Corkill and Walker, 1972; Tanford et al., 1977). The latter retains virtually constant small size ($\bar{m}_w$ = 120) to very high concentration, whereas the former forms large micelles (Figs. 8-2 and 8-3) with the concentration dependence typically observed in the process. One can intuitively imagine that a small difference in oxyethylene chain length (or of temperature) can affect the intimate entanglement of chains in a secondary association process, but it is difficult to see why this factor should have such a profound effect on the ability to form a rodlike *single* micelle, in which the oxyethylene chains are directed radially into the surrounding solvent. There is also direct evidence favoring secondary aggregation, from nmr measurements that show that the ability of the amphiphile to undergo rapid orientational relaxation, at a rate commensurate with the frictional resistance of a small micelle, is unaltered as large particles are formed with increasing concentration (Staples and Tiddy, 1978). The opposite result was obtained for sodium dodecyl sulfate at high ionic strength, and Staples and Tiddy suggested that rodlike primary micelles might actually exist in this system. An alternative explanation is that one is dealing with secondary aggregation, but that the intermicellar forces are stronger than for nonionic micelles, preventing independent motion of the primary micelles.

X-RAY DIFFRACTION. ORDERED STRUCTURES AT HIGH AMPHIPHILE CONCENTRATION

While X-ray diffraction is chiefly useful for the investigation of structures with long-range order, limited information about large particles in solution can be obtained from measurements at small scattering angles. Reiss-Husson and Luzzati (1964,1966) have applied the method to ionic micelles in the absence of added salt, and observed a transition from small globular micelles (spheres and ellipsoids could not be distinguished) to rodlike micelles at very high amphiphile concentration in several of the systems they studied. It is not as difficult to account for the possible stability of rodlike micelles for ionic amphiphiles in the complete absence of added salt as it is at higher ionic strength. While the packing problem remains, the absence of added salt will maximize the coulombic force of repulsion and can raise the optimal surface area sufficiently to allow for formation of rodlike micelles even when simple expressions for W_m, such as given in the previous chapter, are used as basis for calculation (Tanford, 1974).

When the concentration of amphiphile is raised to sufficiently high values (typically 20 to 40% by weight for amphiphiles with a single hydrocarbon chain), ordered liquid crystalline phases are formed. Luzzati and co-workers have studied a number of such systems, and a summary of their results has been presented by Luzzati (1968). The first such phase formed generally represents an ordering of the rodlike micelles already present in solution: the rods are roughly parallel and arranged hexagonally in the plane perpendicular to their length. As the amphiphile concentration is further increased (to 50% by weight and higher), several successive phase transitions occur. The phase structures have not been determined unambiguously, but one of them may be an inverted type of hexagonal phase in which the *solvent* (*water*) occupies roughly cylindrical regions, the remainder of the space being filled by the amphiphile molecules with their head groups at the boundary of the solvent cylinders. As the water content is further depleted, it becomes impossible to maintain a relatively large separation between head groups, and a lamellar phase consisting of extended bilayers is formed. Under completely anhydrous conditions, various crystalline arrays of these bilayers are formed; most of them are stable at high temperatures only.

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NINE

MIXED MICELLES

Because the driving force for association between amphiphile molecules is nonspecific, and because the resultant micelle has a liquidlike core, micelles containing mixtures of amphiphile molecules should be formed readily. If we confine our attention to amphiphiles with linear hydrocarbon tails, there would seem to be no reason for any significant restriction on the nature of the head group in the formation of mixed micelles, other than the fundamental restriction that the interaction between head groups be repulsive. Thus one would not expect to observe formation of mixed soluble micelles when cationic and anionic amphiphiles are mixed in about equimolar amounts. Most other kinds of mixed micelles are in fact readily formed (Shinoda et al., 1963; Becher, 1967), and amphiphile molecules or ions with short hydrocarbon tails, which alone do not form sufficiently large micelles to be recognized as such, are readily incorporated in micelles formed from amphiphile molecules with long hydrocarbon tails.

For the purposes of this book we do not require a rigorous thermodynamic treatment of the formation of mixed micelles, as outlined by Hall and Pethica (1967). Quantitative predictions would in any event be impossible at the present time because the relation between the chemical potential $\mu_{i,\text{mic}}$ of an amphiphile i in a mixed micelle and the corresponding chemical potential in a pure micelle of the same amphiphile depends on knowledge of the repulsive interactions between like and unlike head groups (i.e., W_m of equation 7-14), which we do not now possess.

The fundamental aspects of the formation of mixed micelles can be approached by making the approximation that the micelles constitute a separate phase, and that the free energy of the dispersal of this phase into small particles can be ignored. As has been shown (equations 7-9 and

7-12), this is an excellent approximation for the estimation of the cmc in systems in which the micelle size is large, and still a fairly good approximation for relatively small micelles. When this approximation is made, the only new factor in the thermodynamic treatment of formation of mixed micelles is to make allowance for the mixing of components in the micellar phase. For each component i we have

$$\mu_{i,\text{mic}} = \mu_{i,\text{mic}}^{\circ} + RT \ln X_{i,\text{mic}} + RT\, ln\, f_{i,\text{mic}} \tag{9-1}$$

where $X_{i,\text{mic}}$ is the mol fraction of component i in the mixed micelle, and the activity coefficient reflects nonideality in the mixing process. The standard potential $\mu_{i,\text{mic}}^{\circ}$ is the same as for pure micelles formed by component i. The approximation that micelles are a separate phase is equivalent to assuming infinite micelle size, so that there are no size-dependent terms in the equation. Each component is separately in equilibrium with its monomeric form in the aqueous solution, that is, each $\mu_{i,\text{mic}}$ is equal to the corresponding $\mu_{i,W}$ as given by equation 2-1.

This thermodynamic treatment is analogous to the familiar treatment of liquid-vapor equilibria for liquid solutions. For perfectly ideal mixing, both in the micelle and in the solution, the activity coefficients in equations 2-1 and 9-1 are set equal to unity, and one obtains an equation analogous to Raoult's law for ideal liquid-vapor equilibria,

$$X_{i,W} = X_{i,\text{mic}} X_{i,W}^{\circ} = X_{i,\text{mic}}(\text{cmc})_i^{\circ} \tag{9-2}$$

In this equation $X_{i,W}^{\circ}$ is the free concentration of amphiphile i in equilibrium with pure micelles which, as we have seen, is essentially the same as the critical micelle concentration for pure amphiphile i. Equation 9-2 shows that the concentration of free amphiphile in equilibrium with a mixed micelle will always be smaller than $(\text{cmc})_i^{\circ}$.

Equation 9-2 allows us to construct phase diagrams analogous to those for liquid-vapor equilibria, relating the composition of mixed micelles to the composition of the solution in equilibrium with them. This has been done in Fig. 9-1 for mixtures of two polyoxyethylene-glycol derivatives differing only in the length of the hydrocarbon chain. The ordinate in the figure is the sum of the concentrations of the free amphiphiles in solution, in equilibrium with mixed micelles. This may be regarded as an effective cmc for the mixed micelle and, by equation 9-2, is necessarily a linear function of the mol fraction of either component in the micelle. The composition of the free amphiphile in solution is of course quite different from that of the micelle, being much richer in the component with the larger $(\text{cmc})_i^{\circ}$, the situation being exactly analogous to the enrichment of the vapor phase by the more volatile component of a binary liquid mixture in equilibrium with it. By using the condition that $\sum X_{i,\text{mic}}$ for all compo-

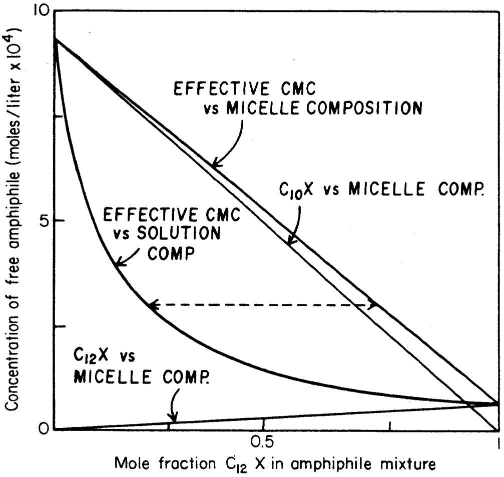

Fig. 9-1. Thermodynamic properties of an ideal mixture of dodecyl hexaoxyethylene glycol monether ($C_{12}X$) with the corresponding decyl compound ($C_{10}X$). The figure gives the concentrations of free $C_{12}X$ and $C_{10}X$ as a function of micelle composition and the sum of these quantities (designated "effective cmc"). The effective cmc is also shown as a function of the composition of free amphiphile in solution. Horizontal lines between the two latter curves provide the composition of free amphiphile in solution in equilibrium with any given composition in the micelle, as is illustrated by the dashed line, which shows that a mixture of free amphiphile in solution containing 80% $C_{10}X$ and 20% $C_{12}X$ is in equilibrium with a micelle containing 28% $C_{10}X$ and 72% $C_{12}X$.

nents in the mixture must be unity, the equation for the effective cmc in terms of monomer concentrations is readily obtained,

$$1/(\text{cmc})_{\text{mixture}} = \sum x_{i,W}/(\text{cmc})_i^\circ \tag{9-3}$$

where $x_{i,W} = X_{i,W}/\sum X_{i,W}$. Since the overall concentration of amphiphile in micellar form is small at the cmc in comparison with $\sum X_{i,W}$, $x_{i,W}$ can be taken to be the mol fraction of component i in the total amphiphile mixture.

The assumption of ideal mixing can be expected to be approximately valid when amphiphiles with the same head group and different alkyl chains are mixed. Experimental data for such mixtures, in fact, agree well with equation 9-3, both for nonionic and ionic head groups (Clint, 1975; Shinoda et al., 1963). The experiments for the ionic amphiphile (Shinoda et al., 1963) were conducted in the absence of added salt and are thus complicated by the effect of the ions of each component in the solution on the value of $(\text{cmc})_i^\circ$ of the other component; correction for this has to be made for each mixture before equation 9-3 is applied. Excellent agreement with equation 9-3 has also been observed for mixtures of $C_{12}H_{25}(EO)_x$ with different values of x (Nishikido et al., 1975). In this case the head groups are not identical, but they differ only in length and not in characteristics that would promote preferential attraction or repulsion between the head groups of the unlike components.

When mixed micelles are formed by amphiphiles with different head groups, ideal mixing is not to be expected. In one example (Lange and Beck, 1973) negative deviations from ideality were observed in mixtures of $C_{12}H_{25}OSO_3^-$ and $C_{12}H_{25}(EO)_8$, that is, cmc values were lower than predicted by equation 9-3, indicating that the average repulsive force in the mixed micelles is less than in micelles formed by the pure components. In such nonideal situations, pseudoideal mixing may occur when one component is in large excess, that is, equation 9-2 would apply to the component in excess and the analog of Henry's law would apply to the other component,

$$X_{j,W} = KX_{j,\mathrm{mic}} \tag{9-4}$$

where K may be smaller or larger than $(\mathrm{cmc})_j^\circ$, depending on the magnitude of the repulsive interaction between the different head groups as compared to that between identical head groups in the pure micelles.

Equation 9-4 would also apply to the incorporation of small amounts of hydrophobic or amphiphilic substances that themselves do not form micelles in micelles of another amphiphile. Combining equation 9-4 with equation 9-2 for the principal component of the micelle leads to the prediction that the addition of any such substance to the solvent medium should lead to a decrease in $X_{i,W}$ and that this decrease should be linearly related to $X_{j,W}$ when the latter is small. An example of this effect is provided by Fig. 9-2.

It is of interest that the slopes of the lines in Fig. 9-2 are considerably smaller than the slopes one would calculate on the basis of the assumption that the alcohol molecules in the micelle are at the same chemical potential as they would be in an ideal liquid solution of alcohols. The slopes expected on the basis of this assumption can be calculated by use of equation 3-3, and are shown as dashed lines in Fig. 9-2. This result is in accordance with the experimental observation that the aliphatic alcohols do not form micelles in aqueous solution, so that the cmc for micelle formation must be larger than the concentration in solution at which phase separation occurs. Nonideality in the mixed micelle may also have an effect, of course, but if this is ignored, the data indicate that $\mu_{\mathrm{mic}}^\circ - \mu_W^\circ$ for formation of hypothetical ROH micelles is about 400 cal/mol more positive than $\mu_{\mathrm{ROH}}^\circ - \mu_W^\circ$ as given by equation 3-3.

It should be noted that virtually all substances containing hydrophobic

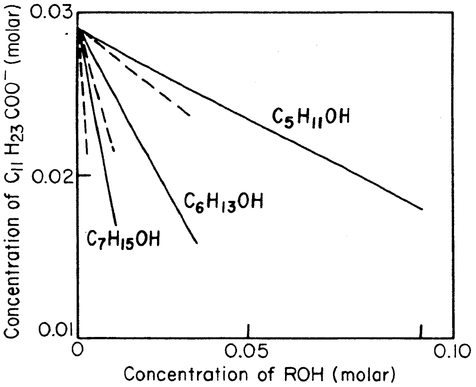

Fig. 9-2. Effect of added alcohol on the concentration of free dodecanoate in equilibrium with its micelles in water at 10°C. The solid lines are experimental lines taken from Shinoda et al. (1963). The dashed lines are calculated with the assumption that μ°_{mic} of an alcohol in the micelle is equal to μ°_{ROH} of pure liquid alcohol.

moieties decrease the concentration of an amphiphile in equilibrium with its micelles. For small molecules (e.g., ethanol) this is generally expressed by stating that their addition to the solvent decreases the cmc. This decrease can be explained only by the incorporation of the added substance into the micelle, since simple addition of the alcohol and similar substances to the solvent must diminish the hydrophobic effect, that is, it would decrease μ°_{W} for the micelle-forming amphiphile and (with no effect on μ°_{mic}) would therefore *increase* the cmc. In fact, an increase in cmc values is observed upon addition of polar molecules, such as urea, which are known to diminish the hydrophobic effect but which could not be incorporated in a micelle with a hydrophobic core (Emerson and Holtzer, 1967).

It is evident from Chapter 8 that the micelle size and especially the formation of large particles by secondary association of small micelles are more sensitive to small changes in head group interactions than the cmc is. Quantitative predictions are not possible, but when nearly ideal mixing occurs one can expect the mixed micelle to be intermediate in size between the corresponding pure micelles. This result has been obtained (Attwood et al., 1971) for mixed micelles formed by $C_{12}H_{25}(EO)_6$ and $C_{16}H_{33}(EO)_9$, even though in this case one of the components is subject to secondary association in the pure state (Fig. 8-3), while the other is not.

IMPORTANCE OF MIXED MICELLES

The ability to form mixed micelles is an inevitable consequence of the mechanism of formation of micelles of any sort, and the results presented here may be taken as confirmation of the principles previously formulated for the formation of single-component micelles, that is, for the liquid state of the micelle core and for the roles of the opposing forces of hydrophobic attraction and head group repulsion. This chapter has been brief because no new principles are involved, but the brevity should not lead one to underrate the importance of mixed micelles. On the contrary, it is probably fair to say that micelle formation per se is a topic of broad interest only because mixed micelles are formed as readily as pure micelles; for example, the incorporation of any molecule with hydrophobic groups into micelles is the basis for the detergent action of amphiphiles. In terms of the focus in the second half of this book on biological membranes, it should be noted that all naturally occurring membranes are mixed micellar aggregates, containing a mixture of various lipids and of proteins with exposed hydrophobic surfaces (Chapter 16). Mixed micelle formation is used directly in the experimental study of membranes, which often begins with the addition of an excess of a simple amphiphile that forms small micelles into which the membrane components can be incorporated (Helenius and Simons, 1975; Tanford and Reynolds, 1976).

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TEN

MONOLAYERS

Amphiphilic molecules or ions in an aqueous solution respond to the hydrophobic effect in another way, by migration to the surfaces of the aqueous medium to form oriented monolayers in which the polar head groups remain in the aqueous medium while the hydrophobic moieties are expelled from it. Such monolayers will generally be present at the water–air interface of any aqueous solution of amphiphiles, and are readily detected by a marked reduction in the surface tension. They will also form at the surface of the container in which the solution is placed, or at the interface between an aqueous solution and an immiscible organic liquid with which it may be placed in contact.

The study of surface monolayers holds an honorable place in the history of science. Surface films can be formed not only by migration from the bulk solution to the surface, but also by the spontaneous spreading of "insoluble" or sparingly soluble liquids placed on a water surface. When this is done with a pure hydrocarbon oil, no spreading occurs, for in the absence of a hydrophilic group there is no driving force for intimate association between the deposited substance and the underlying water (Hardy, 1912). When the oil is amphiphilic, however, rapid spreading occurs as each surface molecule seeks to place its hydrophilic part in the water. The result is the formation of a film that is only one molecule thick. It is of interest to note that the remarkable thinness of such films was observed as early as 1770 by Benjamin Franklin, who noted that "not more than a teaspoonful" of oil (presumably olive oil), placed on a pond at Clapham Common in London, spread spontaneously to cover an area of about half an acre. From this result Franklin could have calculated (but did not) that the final film thickness had to be about 10 Å, which would have been the first reasonably accurate measure of molecular dimensions. (An historical account is given by Giles, 1969.) Rayleigh (1890) calculated a film thickness of 17 Å from a similar experiment on a smaller scale, and subsequent studies of historical importance are those of Traube (1891), which were summarized in Chapter 1, and the work of Langmuir (1917) on the orientation of molecules or ions in surface layers. It is this unique orientation that is responsible for the dramatic decrease in the surface tension of water by surface-active agents, since the surface of a saturated film has the weak intermolecular cohesion characteristic of hydrocarbons instead of the strong cohesion between water molecules.

There are several excellent textbooks on surface chemistry (Adam, 1941; Adamson, 1967; Aveyard and Haydon, 1973; Davies and Rideal, 1961; Harkins, 1952), which provide a wealth of theoretical and experimental information on the subject. Only brief mention of aspects that are of interest in connection with the formation of micelles or membranes will be made here and in later chapters.

FREE ENERGY OF TRANSFER TO AN AIR–WATER INTERFACE

The standard unitary free energy of transfer of amphiphiles from the bulk of a solution to the surface can be calculated from the data of Traube (1891), using the same relation as has been used previously for other transfer processes. Ideality may be assumed both in the solution and in the surface film, since the results extend to low concentrations where only a fraction of the available surface is occupied, and can be extrapolated to infinite dilution (Langmuir, 1917). Under these conditions

$$\mu^{\circ}_{\text{surf}} - \mu^{\circ}_{W} = RT \ln X_W/X_{\text{surf}} \qquad (10\text{-}1)$$

To evaluate the concentration in the surface film (X_{surf}) one has to know how many molecules of amphiphile are in the surface layer and also how thick this layer is, that is, how many water molecules it contains. It is reasonable to assume, in a homologous series, that lowering of surface tension is a measure of X_{surf}, so that *relative* values of $\mu^{\circ}_{\text{surf}} - \mu^{\circ}_{W}$ can be obtained simply from the values of X_W that produce equal lowering of the surface tension. Applying this principle to Traube's results, Langmuir (1917) showed that all of the results, for homologous series of fatty acids, esters, alcohols, and so on, could be represented (in cal/mol) as

$$\mu^{\circ}_{\text{surf}} - \mu^{\circ}_{W} = \text{constant} - 625 n_{\text{C}} \qquad (10\text{-}2)$$

with only the constant term dependent on the head group.

The result given by equation 10-2 is qualitatively consistent with the data for transfer from water to a hydrocarbon solvent given in Chapters 2 and 3. The increment in $\mu^\circ_{HC} - \mu^\circ_W$ per carbon atom is about -850 cal/mol, and the difference between this figure and the increment of -625 cal/mol in equation 10-2 is to be ascribed to the weak cohesive forces between hydrocarbon chains in a liquid medium. When similar calculations are made for adsorption to the interface between an aqueous solution and liquid hydrocarbon, one obtains an increment in $\mu^\circ_{\text{surf}} - \mu^\circ_W$ of -800 to -820 cal/mol per CH_2 group, both for nonionic amphiphiles and for ionic amphiphiles at high ionic strength (Haydon and Taylor, 1960, 1962). This is very close to the values for transfer to bulk hydrocarbon medium (equations 3-2 and 3-3).

PRESSURE-AREA CURVES

Monolayers at an interface can exist in different physical states, analogous to the gaseous, liquid, and solid states of matter in bulk. Information regarding these states and the transitions between them can be obtained by measuring the *surface pressure* of a monolayer as a function of the surface concentration. The surface pressure (Π) is defined as the lateral pressure that must be applied to prevent the film from spreading. The surface concentration may be measured directly on the basis of how much amphiphile has been deposited on the surface. For substances that have a readily measurable solubility in the aqueous solution, the surface concentration may also be determined by means of the Gibbs adsorption equation, which rigorously relates the surface concentration to the effect of bulk concentration on the surface tension. Measurements are made on a large planar surface, and are often reported in terms of plots of Π versus the available area per molecule (A), which is the reciprocal of the surface concentration. This type of plot is particularly useful at high surface pressures, where it defines the minimum area to which a molecule at the surface can be compressed. Illustrative plots are shown in Figs. 10-1 and 10-2.

When the surface is sparsely covered, surface monolayers are "gaseous," that is, they consist of individual adsorbed molecules moving independently. At great dilution their behavior is analogous to an ideal gas and the two-dimensional analog of the ideal equation of state

$$\Pi A = kT \tag{10-3}$$

is obeyed, with k representing Boltzmann's constant. As the pressure is increased, gaslike films become nonideal. If the amphiphile head group is nonionic, an equation of state analogous to the van der Waals equation for

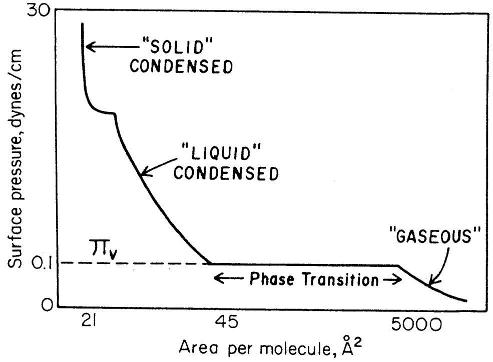


Fig. 10-1. Schematic representation of phase transitions at an air–water interface. Adapted from Harkins (1952). Pressure and area scales are not linear, and numerical values are intended only to indicate typical orders of magnitude.

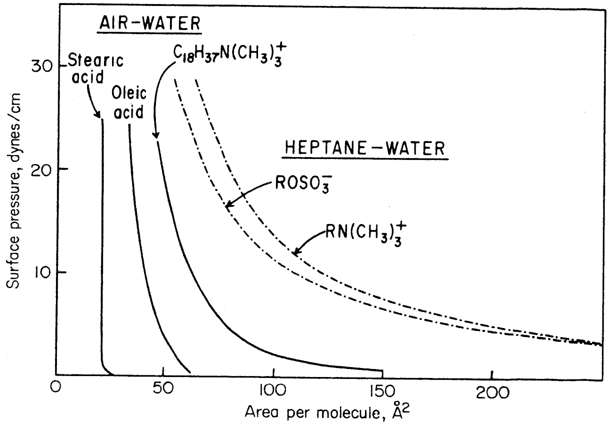


Fig. 10-2. Pressure-area curves at an air–water interface (Davies and Rideal, 1961) and at a heptane–water interface (Brooks and Pethica, 1965). The aqueous phase contained 0.1 *M* NaCl in the experiments with ionic amphiphiles at the heptane interface and 0.5 *M* NaCl for the data on $C_{18}H_{37}N(CH_3)_3^+$ at the air interface.

bulk gases applies, with corrections for the surface area from which an individual adsorbed molecule excludes all others and for the existence of weak attractive forces between molecules. If steric repulsion (as between hard inert spheres) is the only force of interaction, correction need be made only for the excluded area (A_0 per molecule), leading to the Volmer equation of state

$$\Pi(A - A_0) = kT \tag{10-4}$$

This equation is obeyed to surprisingly small areas for alcohols at a water–hydrocarbon interface, with A_0 in the range of 20 to 24 Å^2 (Haydon and Taylor, 1960; Aveyard and Briscoe, 1970). For ionic amphiphiles, however, equation 10-4 does not apply even at a very large molecular areas because of long-range electrostatic repulsions. A number of theoretical equations have been proposed to take electrostatic repulsion into account, but none of them has so far been able to provide a satisfactory fit of experimental data (Haydon and Taylor, 1960, 1962; Mingins et al., 1975).

At an air–water interface the gaslike state is not stable except at very low pressures. As the pressure is increased, the cohesive force between the hydrocarbon chains leads to condensation of the gaslike film to a liquidlike film, as illustrated by Fig. 10-1. This liquidlike film, unlike the bulk liquid state, is highly compressible. The reason for this is that the hydrocarbon chains of the amphiphile can form a coherent film while lying flat on the surface and, with increase in the applied surface pressure, can change their orientation until they are perpendicular to the surface, at a molecular area many times smaller than that of the most expanded liquidlike film. The compression process is not necessarily continuous and two or more distinct "liquid" phases and transitions between them can often be observed (Harkins, 1952, p. 106). At high compression a further transition to a solidlike state may be observed, in which the adsorbed molecules become immobile; this generally requires the existence of an attractive force between head groups in addition to the force of cohesion between hydrocarbon chains. At an interface between an aqueous solution and liquid hydrocarbon, by contrast, the cohesive force between hydrocarbon chains is satisfied at all surface concentrations, and the surface monolayer is normally continuously compressible to very high pressures without a phase transition, even when a simple equation of state cannot be found to relate molecular area to the pressure.

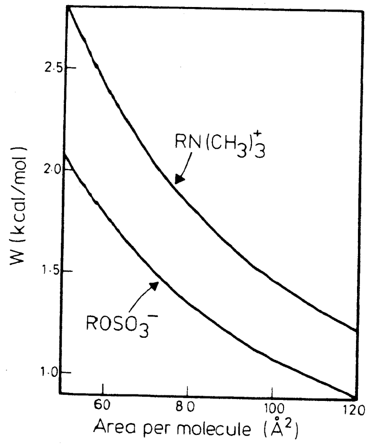
Experimental studies at water–air surfaces have often involved relatively high surface concentrations. Many such studies have employed amphiphile molecules with small head groups that do not repel one another, and therefore do not form micelles in the bulk solution, such as undissociated fatty acids. The results primarily reflect the association tendencies and orientations of hydrocarbon chains, as illustrated for example by the data for stearic and oleic acids in Fig. 10-2. They show that long saturated alkyl chains condense directly (at room temperature) to a solidlike incompressible film, whereas the presence of a double bond, as in oleic acid, leads to a relatively expanded compressible liquidlike film. The limiting surface area at high pressures, for all amphiphiles with single saturated alkyl chains and sufficiently small head groups, is always found to be about 21 Å^2 per molecule, that is, the same as the minimum area per hydrocarbon chain calculated for a bilayer when the hydrocarbon chains are perpendicular to the bilayer surface (p. 55). Liquid films formed by amphiphiles containing mutually repelling head groups are more expanded than the monolayers formed by amphiphiles in which such repulsion is minimal or absent, as illustrated in Fig. 10-2 for an alkyl trimethylammonium salt.

Compression curves at a hydrocarbon–water interface are of greater interest in relation to micelle formation because they provide a measure of the interaction between head groups alone over a wide range of surface area which encompasses the area per head group typically found at surfaces of small micelles (Table 6-2). Unfortunately, most nonionic amphiphiles cannot be studied in this way because of high solubility in the organic solvent, so that no information can be obtained that is relevant to micelles formed by polyoxyethylene derivatives, where an insight into head group interactions would be especially helpful. Illustrative data for an anionic and a cationic amphiphile are given in Fig. 10-2. Brooks and Pethica (1965) have shown that these curves are independent of the alkyl chain length, as is to be expected if interaction between hydrocarbon chains is not a significant factor. (This is of course not true for similar data at an air–water interface because the pressure-area curves in that case depend on the van der Waals attraction between hydrocarbon chains as well as on the repulsion between head groups).

The results of Brooks and Pethica in Fig. 10-2 can be used to calculate the work done in bringing the ionic head groups into close proximity. Since no theoretical equation has yet been found to fit the data, an empirical relation between Π and A has to be used. The total work done in compression is given by $-\int \Pi \, dA$. If there were no repulsion between head groups, the ideal equation 10-3 would apply, and it is the excess over this that gives the work done against the repulsive force, that is, at any final area A,

$$W = -\int (\Pi - kT/A)\, dA \tag{10-5}$$

Results obtained in this way (converted to molar units) are shown in Fig.



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10-3. Because an empirical relation between Π and A was used, no assumption about the origin of the repulsive interaction is involved. Contributions arising from the concentration of neutralizing counterions at the surface (p. 74) and from steric repulsion are thus automatically included.

Figure 10-3 shows that the repulsive force between $—N(CH_3)_3^+$ head groups is greater than that between $—OSO_3^-$, perhaps because counterions can approach closer to the charge of the sulfate group. The result is consistent with the observation that $RN(CH_3)_3^+$ forms smaller micelles than $ROSO_3^-$ under otherwise identical conditions, and it has been used for the quantitative calculation of micelle size and cmc in Chapter 7.

It is necessary to point out here that the determination of accurate pressure-area curves is experimentally difficult, and that the quantitative accuracy of the data of Fig. 10-3 is not established. One of the reasons for this is that surface studies always require extraordinary care because trace impurities are often concentrated at an interface, so that levels of purity in the bulk of a solution that are satisfactory for all other purposes do not guarantee the absence of impurities at an interface. Less easy to overcome are problems arising from the solubility of amphiphiles in the bulk solvent (or solvents) adjacent to the interface. As already noted, this precludes any measurements at a hydrocarbon–water interface for many nonionic amphiphiles with high solubility in organic solvents, but even very low solubility can interfere with the measurements because it can cause a decrease in the amount of amphiphile in the surface monolayer as the surface concentration increases. This creates obvious difficulty in the determination of A, which is the area *per molecule* in the monolayer. With reference to the data of Brooks and Pethica in Fig. 10-2, more recent work from the same laboratory (Taylor and Mingins, 1975) indicates that the data in the figure are not quite correct, the suggested reason being that alcohol which was used as solvent for the application of the very insoluble substances to the surface was not completely removed before measurements were made. However, the newer results are themselves somewhat questionable; they were extended to very low pressure and the extrapolation to the required limit of $\Pi A = kT$ at zero pressure is unsatisfactory (Mingins et al., 1975, Fig. 8).

Phase Transitions

There has been considerable interest in the phase transitions that can be observed at an air–water interface (Fig. 10-1). For example, Gershfeld and Pagano (1972) have measured the "surface vapor pressure" Π_v (defined in Fig. 10-1) for several amphiphiles at different temperatures to obtain values of ΔH and ΔS for the transition from gaslike states to condensed states. It was observed that ΔS for formation of a condensed film (e.g., stearic acid in Fig. 10-2) is considerably more negative than ΔS for formation of an expanded film, presumably reflecting the ordering process in formation of a solidlike structure. The values of ΔS for formation of an expanded film are more positive than one would have expected if contributions from changes in water structure are not taken into consideration. Presumably this reflects the disordering that accompanies displacement of water molecules from the surface, as discussed in Chapter 5.

Other Physical Measurements

In addition to surface pressure, other physical properties of surface films have been studied as a function of surface concentration. They include measurements of the electrical potential across a monolayer, which provides information on the orientation of the adsorbed molecules, measurements of surface viscosity, and optical measurements. The techniques employed and the available results are described in the references given at the beginning of this chapter.

MIXED MONOLAYERS

Monolayers, like micelles, may contain mixtures of amphiphiles. Pressure-area curves of such mixed films have to be interpreted with great caution, because more than one liquidlike phase of different composition may be present at the same time (Gaines, 1966). An additional difficulty, which applies to all surface films, but is an especially important possible source of error for mixed monolayers, is that equilibration between the monolayer and the bulk solution may be very slow. For mixed monolayers the equilibration process may influence the monolayer composition, which may therefore change with time in an experiment in which (as is often done) the monolayer constituents are spread upon the surface without stirring or other means of accelerating the equilibration process.

Experimental pressure-area data for mixed liquidlike films have been obtained at air–water surfaces, and they indicate that interactions between surface components are often far from ideal. Both "condensation" and "expansion" have been observed, that is, the area per adsorbed molecule may be less than or greater than the mean area calculated for the same pressure on the basis of the pressure-area curves of the pure components. Since orientation of the hydrocarbon chains is a primary determinant of surface area (except at the high pressure limit), it is not surprising to find that the lengths of alkyl chains have an important influence on the observed results.

It has been suggested (Pagano and Gershfeld, 1972) that measurements of the equilibrium surface vapor pressure (Π_v) at the transition between gaseous and liquidlike films provide a more readily interpretable means of investigating mixed monolayers. This phenomenon obeys the same thermodynamic laws as apply to the vapor pressures of bulk solutions. Pagano and Gershfeld have shown that some mixtures behave ideally by this criterion and others show positive deviations from ideality. Negative deviations, corresponding to a greater mutual attraction between disparate molecules than between the molecules of the pure components, were not observed in the limited number of systems they investigated. Some of the systems studied by Pagano and Gershfeld gave Π_v versus composition curves indicative of incompletely miscibility of the components, that is, two liquidlike phases appeared to coexist at equilibrium over some part of the composition range. The latter result reinforces the cautionary note expressed above with regard to pressure-area curves: their interpretation is not clear if two distinct liquidlike phases are present.

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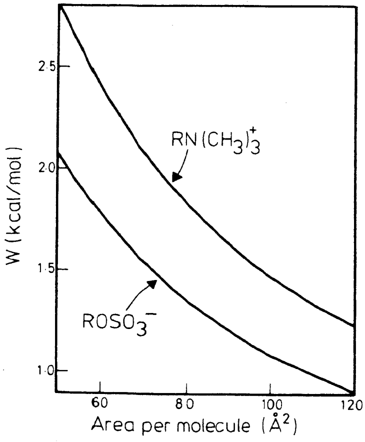

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Monolayers, like micelles, may contain mixtures of amphiphiles. Pressure-area curves of such mixed films have to be interpreted with great caution, because more than one liquidlike phase of different composition may be present at the same time (Gaines, 1966). An additional difficulty, which applies to all surface films, but is an especially important possible source of error for mixed monolayers, is that equilibration between the monolayer and the bulk solution may be very slow. For mixed monolayers the equilibration process may influence the monolayer composition, which may therefore change with time in an experiment in which (as is often done) the monolayer constituents are spread upon the surface without stirring or other means of accelerating the equilibration process.

Experimental pressure-area data for mixed liquidlike films have been obtained at air–water surfaces, and they indicate that interactions between surface components are often far from ideal. Both "condensation" and "expansion" have been observed, that is, the area per adsorbed molecule may be less than or greater than the mean area calculated for the same pressure on the basis of the pressure-area curves of the pure components. Since orientation of the hydrocarbon chains is a primary determinant of surface area (except at the high pressure limit), it is not surprising to find that the lengths of alkyl chains have an important influence on the observed results.

It has been suggested (Pagano and Gershfeld, 1972) that measurements of the equilibrium surface vapor pressure (Π_v) at the transition between gaseous and liquidlike films provide a more readily interpretable means of investigating mixed monolayers. This phenomenon obeys the same thermodynamic laws as apply to the vapor pressures of bulk solutions. Pagano and Gershfeld have shown that some mixtures behave ideally by this criterion and others show positive deviations from ideality. Negative deviations, corresponding to a greater mutual attraction between disparate molecules than between the molecules of the pure components, were not observed in the limited number of systems they investigated. Some of the systems studied by Pagano and Gershfeld gave Π_v versus composition curves indicative of incompletely miscibility of the components, that is, two liquidlike phases appeared to coexist at equilibrium over some part of the composition range. The latter result reinforces the cautionary note expressed above with regard to pressure-area curves: their interpretation is not clear if two distinct liquidlike phases are present.

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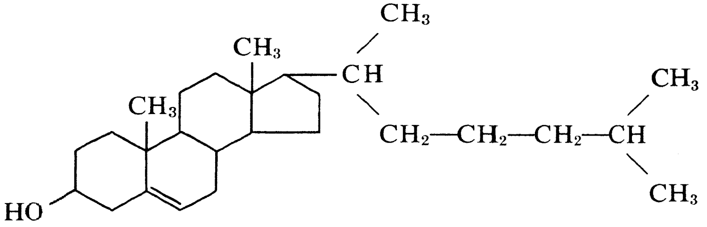
ELEVEN

BIOLOGICAL LIPIDS

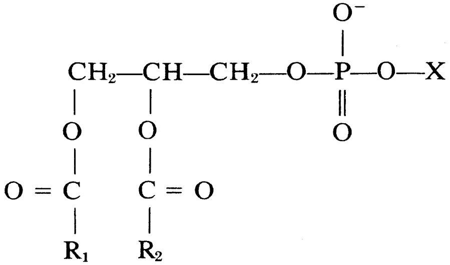
The biochemist uses the term "lipid" to define all organic molecules of biological origin that are highly soluble in organic solvents and only sparingly soluble in water. Included in this definition are fats, some hormones and vitamins, and many other substances of diverse chemical identity and biological function. The discussion in this chapter will be confined to lipids present in biological membranes or in soluble lipoprotein complexes, and they fall into two classes:

1. Amphiphile molecules or ions of the type discussed previously in this book, containing a strongly hydrophilic head group and one or more (most often *two*) long hydrocarbon tails.
2. Cholesterol and acyl esters of cholesterol. These substances do not resemble any we have previously considered; their principal constituent is pure hydrocarbon in the form of the relatively rigid steroid ring. Cholesterol, the structural formula for which is



may be considered amphiphilic by virtue of possession of a single OH group. In cholesteryl esters this OH group forms an ester link with a fatty acid. In either case the affinity for water is very weak compared to that of the strongly hydrophilic head groups of other membrane lipids.

The most abundant lipids in the first category contain two hydrocarbon chains attached to a single polar head group. Among lipids of this type are most of the phosphoglycerides, which have the general formula



$$\begin{array}{cccccccc} & & & & & & O^- & \\ & & & & & & \mid & \\ & CH_2 & - & CH & & -CH_2-O- & P & -O-X \\ & \mid & & \mid & & & \Vert & \\ & O & & O & & & O & \\ & \mid & & \mid & & & & \\ O= & C & & C & =O & & & \\ & \mid & & \mid & & & & \\ & R_1 & & R_2 & & & & \end{array}$$

(I)

where R_1 and R_2 are hydrocarbon chains. The head group bears a net negative charge at neutral pH if the X group in this formula is neutral or zwitterionic, as follows:

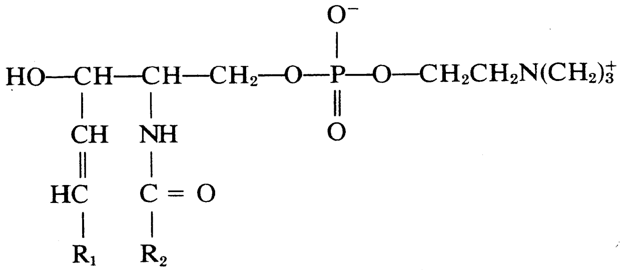
Phosphatidic acid $-X = -H$
Phosphatidylserine $-X = -CH_2CH(NH_3^+)COO^-$
Phosphatidylinositol $-X = -C_6H_6(OH)_5$
Phosphatidylglycerol $-X = -CH_2CHOHCH_2OH$

Alternatively the head group may be zwitterionic at neutral pH, if the X group bears a positive charge, as in

Phosphatidylethanolamine $-X = -CH_2CH_2NH_3^+$
Phosphatidylcholine $-X = -CH_2CH_2N(CH_3)_3^+$

Aminoacyl derivatives of phosphatidyl glycerol occur in some bacteria (Houtsmuller and van Deenen, 1965; Lennarz, 1972). Included in this category is the lysyl ester of phosphatidylglycerol, which bears two positive charges, conferring a net positive charge on the head group as a whole. Apart from this instance, phosphoglycerides with positively charged head groups are rare or nonexistent.

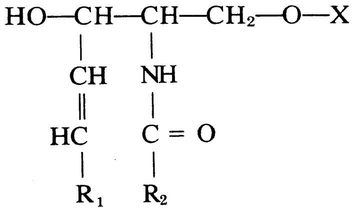
Plasmalogens represent a variant form of I in which one of the ester links is replaced by an ether linkage, and similar molecules with two ether linkages have also been observed. Another lipid, chemically quite distinct but geometrically similar, is sphingomyelin:



$$HO-\underset{\substack{|\\ CH\\ \|\\ HC\\ |\\ R_1}}{CH}-\underset{\substack{|\\ NH\\ |\\ C=O\\ |\\ R_2}}{CH}-CH_2-O-\overset{\substack{O^-\\ |}}{\underset{\substack{\|\\ O}}{P}}-O-CH_2CH_2N(CH_2)_3^+$$

(II)

Most glycolipids are also sphingolipids, with the general formula



$$HO-\underset{\substack{|\\ CH\\ \|\\ HC\\ |\\ R_1}}{CH}-\underset{\substack{|\\ NH\\ |\\ C=O\\ |\\ R_2}}{CH}-CH_2-O-X$$

(III)

The carbohydrate moiety X may be a single neutral hexose sugar (as in cerebrosides), a sulfated sugar (cerebroside sulfate or sulfatide), or a more complex oligosaccharide. The head group of sulfatides bears a negative charge, as does the head group of gangliosides, in which X is a complex oligosaccharide containing one or more mols of sialic acid. Glycolipids are especially abundant in the brain, and explicit formulas for many of them can be found in a review by Ramsey and Nicholas (1972).

Mitochondria contain considerable quantities of cardiolipin, in which *four* hydrocarbon chains are attached to a single head group. This lipid is essentially a dimeric form of I. Two molecules of the diacyl glycerophosphate moiety are linked by one molecule of glycerol (i.e., —X is replaced by —$CH_2CHOHCH_2$—). There are two negative charges on the head group.

Lipids with a single hydrocarbon chain per head group occur in only very small quantities.[1] Lipids in this category are the lysophospholipids (formula I with one of the acyl group removed) and the free fatty acid anions. As noted in Chapter 9, all hydrophobic or amphiphilic molecules tend to be incorporated in micelles formed by other amphiphiles and, considering membranes tentatively as having some resemblance to micelles formed by the principal membrane lipids, the presence of some minor components, such as fatty acid anions, may simply be a reflection of their presence in the medium with which the membrane is in contact.

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[1] The membrane of the hormone-secreting granules of the adrenal cortex constitutes an exception; it has been shown to contain about 15% of lysophosphatidylcholine (Winkler and Smith, 1968). A similar high content of lysophosphatidylcholine in zymogen-secreting granules of the pancreas has been shown, however, to be an artifact, arising from enzymatic degradation during the isolation procedure (Meldolesi et al., 1970).

Analytical data for the lipid content of several membranes are shown in Table 11-1. They are intended primarily to show that composition with respect to lipid classes is quite different for different membranes. The common features of all the data are (1) that 75% or more of membrane

Table 11-1. Lipid Compositions of Some Biological Membranes[a] (expressed as percent by weight of total lipid)

	Human Erythrocyte[d]	Human Myelin[e]	Beef Heart Mitochondria[f]	E. Coli[g]
Phosphatidic acid	1.5	0.5	0	trace
Phosphatidylcholine[b]	19	10	39	0
Phosphatidylethanolamine[b]	18	20	27	65
Phosphatidylglycerol	0	0	0	18
Phosphatidylinositol	1	1	7	0
Phosphatidylserine	8.5	8.5	0.5	trace
Cardiolipin	0	0	22.5	12
Sphingomyelin	17.5	8.5	0	0
Glycolipids	10[h]	26[i]	0	0
Cholesterol[c]	25	26	3	0

[a] Quantitative lipid analysis is a rapidly developing science, and many of these figures are likely to change as it progresses. In bacterial membranes, lipid composition depends to some extent on growth phase and nutritional factors.

[b] Up to one-third of the phosphatidyl derivatives may be in the plasmalogen form, that is, with an ether rather than an ester link between the hydrocarbon chain and the glyceryl moiety. For our purposes the distinction is not important.

[c] The figures are for free cholesterol. Cholesterol esters occur in soluble lipproproteins, but have not been found in membranes.

[d] Rouser et al. (1968).

[e] Dickerson (1968), Rouser et al. (1968). The values given are for central nervous system myelin.

[f] Rouser et al. (1968). The values given are for total mitochondrial lipid. Inner and outer membranes differ significantly in composition.

[g] Ames (1968), Cronan and Vagelos (1972). Data are for total lipid, including both inner and outer membranes.

[h] 80% of the glycolipid is cerebroside, 20% is sulfatide.

[i] Principally polyhexosides and gangliosides.

lipid consists of amphiphilic molecules with two hydrocarbon chains per head group, (2) that about 20 to 30% of the lipid contains anionic head groups, and (3) that there are no cationic head groups at all, those that are not anionic being neutral or zwitterionic. (As noted above, positively charged head groups do occur in some bacterial membranes.)

The hydrocarbon chains of biological lipids are invariably very long. Only small amounts of fatty acids with fewer than 16 carbon atoms are usually obtained upon hydrolysis of diacyl lipids, and chain lengths to 24 carbon atoms occur frequently. An almost universal feature is the presence of unsaturated fatty acids,[2] typically to the extent of about 50% of the total fatty acid content. In fact, individual molecular species more often than not contain one chain of each kind on the molecule; in phosphoglycerides (formula I) from many sources R_1 tends to be a saturated fatty acyl group and R_2 an unsaturated one (van Deenen, 1971). However, di-saturated and di-unsaturated species occur as well; for example, dipalmitoyl phosphatidylcholine represents about 10% of the total phosphatidylcholine in the human erythrocyte membrane.[3] It should also be noted that the fatty acid composition of the microorganism *Acheloplasma laidlawii B* can be altered dramatically by regulation of the growth medium, and the organism is viable with a saturated fatty acid content of as much as 90% (McElhaney and Tourtelotte, 1969). Similar variations in fatty acid content can be induced in mutant bacteria lacking enzymes for fatty acid synthesis. Unsaturated fatty acids may contain up to six double bonds. Double bonds in unsaturated fatty acids from bacterial or animal membranes are usually in the *cis*-configuration and, in polyunsaturated acids, tend not to be conjugated. Reviews by Rouser et al. (1968) and van Deenen (1971) may be consulted for additional information.

All of the biological lipids described above, except cholesterol and its esters, resemble the simpler amphiphiles discussed in preceding chapters. They are expected to form micelles in aqueous media and, because their constituent hydrocarbon chains are invariably very long, the concentration of unassociated lipid in equilibrium with the micelles (which we shall continue to assume is essentially the same as the critical micelle concentration) should be very small. For the predominant type of molecule, containing two hydrocarbon chains per head group, the micelle is expected to be of the bilayer type, and there should be a tendency for the bilayers to form closed vesicles (Chapter 6). Mixtures of lipids should readily form mixed micelles and, if most of the constituent molecules contain two hydrocarbon chains per head group, the mixed micelles should also be bilayers. All available experimental data, some of which will be summarized in this chapter, support these predictions.

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[2] In some bacterial lipids a methylene group may be added across the unsaturated bond to produce a cyclopropyl group, or branched saturated fatty acids may replace unsaturated fatty acids. The important factor is geometrical and not chemical: about half the acyl chains must have structures that prevent them from forming parallel close-packed arrays with linear saturated hydrocarbon chains.

[3] Dipalmitoyl phosphatidylcholine is a major constituent of the lipids of lung tissue (van Deenen, 1971), but most of it is probably derived from a specific lipoprotein present there and not from phospholipid membranes.

In considering the physicochemical data to be presented we should keep in mind the likelihood, as discussed in Chapter 6, that micellar structures formed by amphiphile molecules with very long *saturated* hydrocarbon chains may have hydrophobic cores in which the hydrocarbon chains are in an ordered paracrystalline array at room temperature, instead of being liquid. For molecules with *unsaturated* hydrocarbon chains, on the other hand, the melting point for ordered structures is expected to be below room temperature, and the hydrophobic cores may accordingly be in a fluid state. The data to be presented in this and the following chapter will in fact demonstrate that ordered and disordered hydrophobic cores exist much as expected, and the results of the following chapter will permit us to refine our concepts of the meaning of "order" and "disorder" in these systems.

Many of the results to be discussed were obtained with lipids isolated from natural sources, containing molecules with a mixture of hydrocarbon chains. The material of this type that has been employed more frequently than any other is egg yolk phosphatidylcholine. This material is pure with regard to the head group, but contains a mixture of acyl chains, about 50% of them saturated and 50% unsaturated. Principal constituents after hydrolysis of the ester linkage were found in a typical analysis (Huang et al., 1964) to be palmitic acid, stearic acid, oleic acid (one double bond), and linoleic acid (two double bonds). A small amount of $C_{19}H_{32}COOH$ (four double bonds) was also present. A typical individual molecule in the mixture probably contains one saturated and one unsaturated hydrocarbon chain, as mentioned earlier. Ordered arrays of the hydrocarbon chains have low stability in a mixture of this kind, and egg phosphatidylcholine will be seen to resemble pure synthetic molecules with unsaturated hydrocarbon chains in that it forms bilayers with a liquid hydrophobic core at room temperature. In terms of a number of physical properties not discussed here, the egg yolk product resembles pure dioleoyl phosphatidylcholine (DeGier et al., 1968).

CRITICAL MICELLE CONCENTRATION

The cmc values for the phospholipids of major interest are very small and therefore difficult to measure. Table 11-2 contains the reliable mea-

Table 11-2. **Critical Micelle Concentrations of Biological Lipids in Aqueous Solution at 25°C**

	cmc(mol/liter)			
	Observed	Calculated		$\mu^{\circ}_{\text{mic}} - \mu^{\circ}_{W}$ (kcal/mol)[i]
		(betaines)[h]	(glucosides)[h]	
Palmitoyl[a] lysophosphatidylcholine[b]	7×10^{-6}	6×10^{-5}	5×10^{-6}	−9.2
$C_{15}H_{31}$ deoxyether analog[c]	6×10^{-6}			—
$C_{15}H_{31}$ phosphorylcholine[d]	3×10^{-5}			—
		Calculated (lysophosphatidylcholine)[j]		
Diheptanoyl[a] phosphatidylcholine[e]	1.4×10^{-3}	3×10^{-3}		−6.1
Dioctanoyl[a] phosphatidylcholine[e]	2.7×10^{-4}	5×10^{-4}		−7.3
Didecanoyl[a] phosphatidylcholine[f]	5×10^{-6}	1.2×10^{-5}		−9.6
Dipalmitoyl[a] phosphatidylcholine[g]	4.7×10^{-10}	2×10^{-10}		−15.1

[a] One carbon atom of an acyl chain is part of the ester link and not part of the hydrocarbon chain, that is, the palmitoyl moiety has a C_{15} chain, and so on.

[b] Haberland and Reynolds (1975).

[c] Weltzien et al. (1977). Extrapolated from results for C_{10}, C_{12}, and C_{14} chains.

[d] Hayashi et al. (1972). Interpolated from results for C_{10}, C_{12}, C_{14}, and C_{16} chains.

[e] Tausk et al. (1974a).

[f] Reynolds et al. (1977).

[g] Smith and Tanford (1972).

[h] Based on extension of the straight lines of Fig. 7-3 for alkyl betains or alkyl glucosides.

[i] Calculated by equation 7-11, with the cmc in mol fraction units and $\ln f_W = 0$.

[j] Based on the chain-length dependence for the deoxyether analogs, with the assumption that the addition of the second chain is equivalent to lengthening one chain by 60% of the alkyl chain length (Fig. 7-4).

surements that have been reported for natural phospholipids and for two analogs of lysophosphatidylcholine, a deoxyether analog in which the ester link to the phosphoglyceride moiety is replaced by an ether link, and a phosphorylcholine which has the alkyl chain attached directly to the phosphate group. The two analogs were studied as a function of hydrocarbon chain length, and linear relations with the same slope as for other single chain amphiphiles (Fig. 7-3) were obtained. Since the head group in all of these compounds is zwitterionic, one might have expected the absolute cmc values to correspond to the data in Fig. 7-3 for *N*-alkyl betaines, but the observed results for palmitoyl lysophosphatidylcholine and for the series of ether analogs are an order of magnitude lower and correspond closely to the results in Fig. 7-3 for nonionic amphiphiles such as the alkyl glucosides. The reason for this is presumably the fact that the zwitterionic group in these molecules lies at a considerable distance from the alkyl chain because of the intervening glyceryl moiety. The cmc values for alkyl phosphorylcholines, where the hydrocarbon chain is attached directly to the zwitterionic group, are much closer to those for *N*-alkyl betaines.

The cmc values in Table 11-2 for diacyl phosphatidylcholines can be predicted tolerably well if we apply the principle suggested by Fig. 7-4, that a second hydrocarbon chain attached to a single head group exerts an effect equivalent to about 60% of that which would result from extending a single chain by the same number of carbon atoms. For example, the calculated cmc shown for dipalmitoyl phosphatidylcholine (two C_{15} chains) is the extrapolated cmc for the C_{24} single-chain compound.

It is likely that the hydrocarbon chains of dipalmitoyl phosphatidylcholine are in an ordered paracrystalline array at 25° instead of in a liquid core. This should not have a large effect on the cmc. The free energy of melting of the ordered array is probably less than 1 kcal/mol and the difference between the cmc of otherwise identical micelles with ordered and liquid cores should thus be less than a factor of 5. This difference is not much greater than the precision with which the cmc can be measured: the probable error is about a factor of two.

SOLUBLE MICELLES AND VESICLES

The micelles formed by lysophosphatidylcholines and their analogs are small and globular, similar to those observed for simpler single-chain amphiphiles. Weltzien et al. (1977), for example, obtained $\bar{m} = 70$ for the deoxyether analog with a C_{12} chain and $\bar{m} = 140$ with a C_{16} chain. Saunders (1966) obtained $\bar{m} = 180$ for the mixed product obtained by enzymatic cleavage of egg yolk phosphatidylcholine. The intrinsic viscosity of the latter was found to be 4 cc/g. Haberland and Reynolds (1975) found evidence for secondary association with increasing concentration in their work with palmitoyl lysophosphatidylcholine.

Phospholipid molecules containing two hydrocarbon chains form small micelles only when the hydrocarbon chains are extremely short. Tausk et al. (1974b) studied diacyl phosphatidylcholines and observed well-behaved small micelles only for the dihexanoyl compound (C_5 chains). Small micelles formed by the diheptanoyl compound were subject to secondary association, and extensive aggregation and phase separation occurred with the dioctanoyl compound. Diacyl phospholipids with the longer hydrocarbon chains normally found in nature are difficult to disperse in aqueous solution at all. When water is added to dry phospholipid, swelling takes place, with formation of liquid crystalline phases of different kinds (see below). Further addition of water tends to lead to dispersal of this lipid without disruption of the ordered aggregated structures. This is probably due to the tendency of bilayers to form closed structures in the presence of water. Electron micrographs of phospholipid preparations containing water or of discrete "liposomes" obtained from them (Dervichian, 1964; Bangham et al., 1965) invariably show multilayered closed structures, such as illustrated by Fig. 11-1, which cannot be further subdivided without rupture of the individual layers.

As was first observed by Saunders et al. (1962), small soluble particles can be obtained from multilayered structures by ultrasonic radiation. The particles so formed are in fact closed vesicles, some still bounded by several bilayers, but others bounded by only a single bilayer, as in Fig. 11-1*b*. Relatively homogeneous preparations of single-walled vesicles can be separated from the sonicated mixture by gel exclusion chromatography, and vesicles formed in this way from egg yolk phosphatidylcholine have been carefully characterized by Huang (1969); subsequent refinements in the measurements are summarized by Huang and Mason (1978). The vesicles were found to be nearly uniform in size, with a molecular weight of 1.9×10^6, corresponding to $\overline{m} = 2400$. Hydrodynamic and several other kinds of measurements indicate that the vesicles are close to spherical, with an external diameter of 210 Å and an internal solvent-filled cavity having a diameter of about 100 Å, which gives a bilayer thickness of 50 Å. (If these dimensions are measured with the aid of nmr probes that penetrate into the hydrated head group layer, the apparent thickness of the bilayer is diminished; the external and internal diameters become 198 and 124 Å, respectively.) Vesicles with remarkably similar dimensions have been formed from the pure lipid, dipalmitoyl phosphatidylcholine, above the temperature at which the saturated hydrocarbon chains of this lipid tend to form ordered paracrystalline arrays (Chrzeszczyk et al., 1977).

More recent work (Batzri and Korn, 1973; Brunner et al., 1976) has shown that small single-walled vesicles are readily formed by techniques not involving sonication, for example, by dissolving phospholipid in a detergent and then slowly dialyzing away the detergent. Vesicles have been prepared from phospholipids with a variety of head groups, and even from molecules with two long saturated hydrocarbon chains at a temperature at which the hydrocarbon chains would have been expected to be in an ordered paracrystalline array not compatible with formation of a curved surface (Sheetz and Chan, 1972; Suurkusk et al., 1976). It would appear from these results that small vesicles are the thermodynamically favored state of aggregated phospholipids, formed whenever conditions for dynamic equilibration prevail. This result was predicted on the basis of the geometrical considerations of Table 6-2, but it was pointed out there that the calculations treated the bilayer as a structureless fluid, and they did not take into consideration the molecular nature of the constituent phospholipid molecules and the difficulty of packing them into a spherical shell.

The packing problem has been examined in detail by Chrzeszczyk et al. (1977) and Huang and Mason (1978). Though the *overall* thickness of the bilayer and the *average* area per head group in the vesicles studied by these investigators are consistent with results obtained from X-ray diffraction of planar phospholipid bilayers (see below), the arrangement of the lipid molecules in the vesicle bilayer must clearly be quite different from that in a planar bilayer. The high curvature of the vesicle wall makes the external area of the bilayer three times as large as the internal area, and it causes the hydrocarbon chains entering from the external side to extend into a space of continuously decreasing radius, while those entering from the inside extend into a radially expanding space. As a result of this unavoidable asymmetry, there are more than twice as many lipid molecules in the outer half of the bilayer than in the inner half. The outer half is thicker than the inner half because the increasing constriction forces the hydrocarbon chains to be more fully extended, and the area per head group is substantially larger on the outside than on the inside. It is quite remarkable that these difficulties in packing do not interfere with vesicle formation, and it provides support for use of a model with a structureless hydrophobic core in the thermodynamic consideration of the formation of micellar aggregates of all kinds, as was done in earlier chapters.

Vesicles have been formed from mixtures of phospholipids with different head groups. The outer and inner halves of the bilayer have different

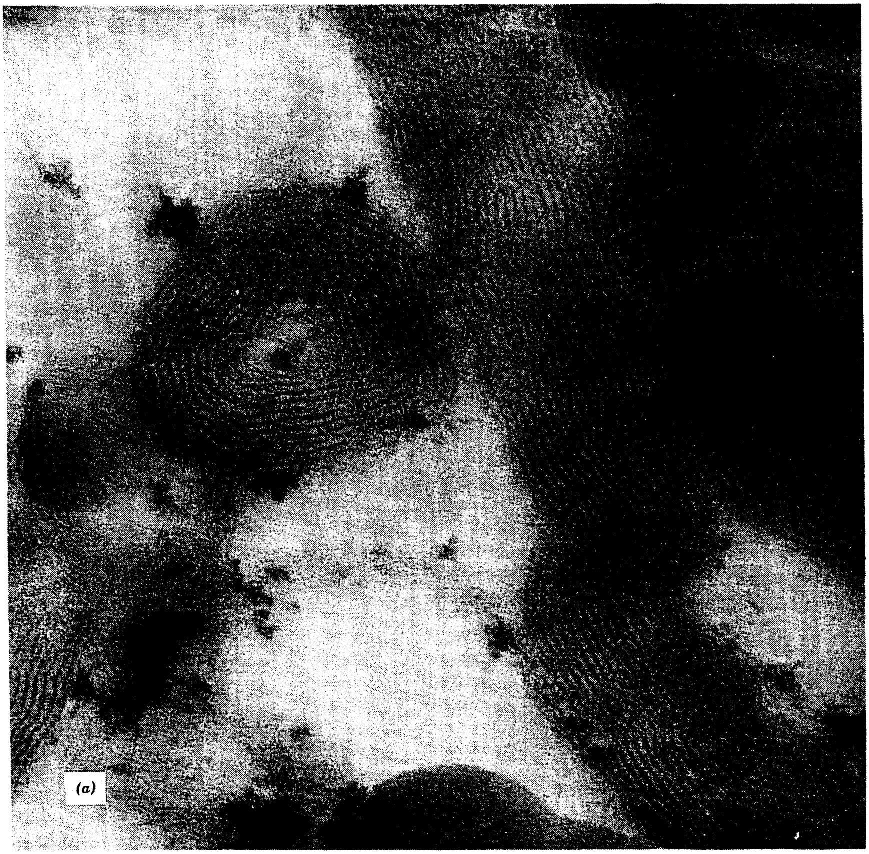

Fig. 11-1. Typical electron micrographs of phospholipid preparations. (*a*) In the form of extended bilayers and multiwalled vesicles (reprinted with permission of Pergamon Press, Ltd., from Dervichian, 1964).

compositions when this is done, that is, the head groups that might be expected to prefer a larger surface area tend to be preferentially in the outer half. For example, the charged head group is preferentially on the outside in vesicles formed from a mixture of a charged and a zwitterionic lipid (Michaelson et al., 1973), and the larger choline head group is preferentially on the outside in vesicles formed from a mixture of diacyl phosphatidylcholine and diacyl phosphatidylethanolamine (Litman, 1974).

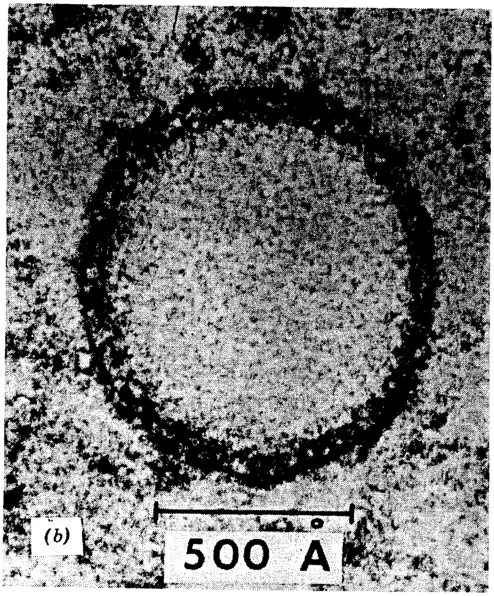

Fig. 11-1. *(Continued)* (*b*) In the form of a single-walled vesicle (taken from Miyamoto and Stoeckenius, 1971). The vesicle shown in this picture is much larger than the small single-walled vesicles discussed in the text.

It is of interest to note that the hydrocarbon layer at the center of a bilayer, though only about 30 Å thick, provides an effective barrier to ions and to highly polar substances that are not significantly soluble in hydrocarbon. Bilayer-bounded vesicles are virtually impermeable to small cations and to most large polar molecules (Hauser et al., 1973; Toyoshima and Thompson, 1975); they are slightly permeable to Cl^-, but the mechanism may involve the transient formation of a neutral species such as HCl. The solubility of water in liquid hydrocarbon is appreciable (Finkelstein, 1976) and water permeability is therefore relatively high, so that the internal space of vesicles is in osmotic equilibrium with the external solution. Vesicles prepared in one solution and transferred to an aqueous solution of different composition can maintain their original internal composition for a long time, provided that the two solutions have the same osmotic pressure. (If the formation of vesicles is a thermodynamic phenomenon, as the data cited above indicate, we have here a curious paradox: phospholipid molecules, in seeking their own equilibrium state, create a structure that *prevents* equilibration of other solutes in the solution.)

LIQUID CRYSTALLINE PHASES AT HIGH LIPID CONCENTRATIONS

As was true for simple amphiphiles (Chapter 8) biological lipids form a variety of ordered phases (liquid crystals) in mixtures with water when the lipid content is high. As previously noted, these structures tend to persist even when large amounts of water are added, unless disrupted by sonication. In the case of simple amphiphiles the molecular arrangement within the micellar aggregates is greatly altered as the water content is reduced, but for lipids with two hydrocarbon chains per molecule the bilayer arrangement is a common feature of all structures that have been reported, and structural studies of the ordered phases are therefore of special interest because they permit determination of bilayer dimensions.

Numerous studies have been carried out by electron microscopy (Fig. 11-1) and X-ray diffraction. The pioneering work by X-ray diffraction was done by Bear et al. (1941) and Palmer and Schmitt (1941). They studied a variety of lipids from natural sources, and were able to demonstrate the existence of the bilayer structure and give essentially correct dimensions for it. In particular, they were the first to observe, in anhydrous or nearly anhydrous preparations, the spacing of 4.2 Å, characteristic of hexagonally packed hydrocarbon chains perpendicular to the bilayer plane, and to show the broadening of this spacing to an average value of 4.6 Å when water was added. They correctly inferred from this that the interior of the bilayer must then be in a liquidlike state (Schmitt, 1939). More detailed X-ray studies were subsequently made by Luzzati and co-workers, and most of this work has been summarized in a review by Luzzati (1968). Phospholipids with a variety of head groups have been shown to form similar multilamellar structures (Reiss–Husson, 1967; Papahdjopoulos and Miller, 1967), including the phospholipid mixture from mitochondria (Gulik–Krzywicki et al., 1967), which has a high content of cardiolipin (four hydrocarbon chains per head group). The latter finding is not surprising since the cardiolipin molecule is actually structurally equivalent to two normal phosphoglyceride molecules linked together.

The most accurate bilayer dimensions are probably those given by Levine et al. (1968) and by Levine and Wilkins (1971) who obtained highly ordered stacks of bilayers, separated by water layers, by several techniques. The advantages of such a system are obvious: discrete Bragg reflections are observed in X-ray diffraction instead of continuous diffraction envelopes, and the spacings that they represent are automatically oriented with respect to the direction of the bilayer. Although the overall resolution is relatively poor owing to the rather limited degree of order, a sufficient number of reflections were obtained in the direction perpendicular to the bilayer surface to permit analysis by Fourier synthesis to

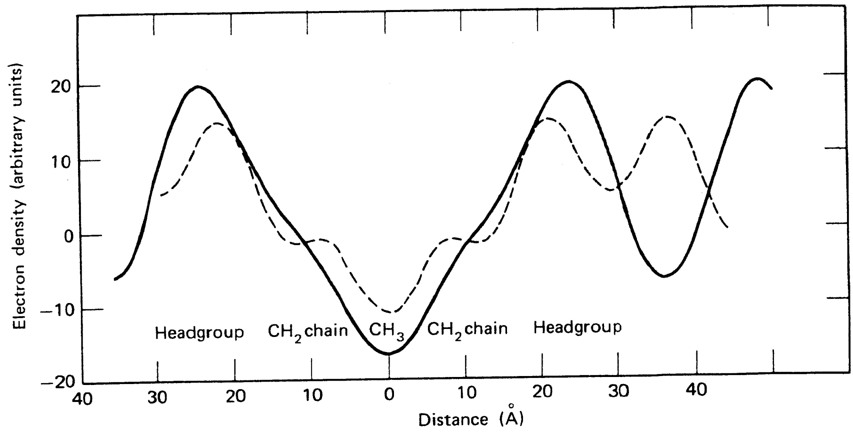

Fig. 11-2. Electron density profile of one repeat of an oriented stack of bilayers of dipalmitoyl phosphatidylcholine (taken from Levine et al., 1968). Electron densities are given relative to liquid water as zero. The dashed line represents lipid in the dry state, and the solid line represents data obtained when the system is under water.

obtain an electron density profile in that direction. The phase assignment required for this procedure was based on the reasonable assumption that the changes in the repeat distance between bilayers, obtained by swelling the sample with water, are accompanied by relatively small differences in the width of an individual bilayer.

Figure 11-2 represents one repeat of the electron density profile across the stacked bilayers for dry and wet dipalmitoyl phosphatidylcholine, and the interpretation is virtually self-explanatory. The peaks of high electron density must represent the location of phospholipid head groups (which contain the electron dense acyl and phosphate ester groups). Two of these peaks are separated by about 16 Å in the dry state, and they must represent head group locations of adjacent bilayers because the separation increases to 25 Å in the wet state. The other two peaks, separated by about 43 Å in the dry state, must represent head group locations on either side of a single bilayer: the increase in their separation in the wet state occurs *outside* of the dry state peaks and represents an extension of the head groups toward the outside, with intercalation of water molecules, and does not involve an increase in the width of the hydrophobic core. The most striking aspect of the profile is the dip in the center of this region, where the electron density falls well below that of water. This dip has been interpreted as representing the location of the methyl groups at the ends of the hydrocarbon chains. Not being covalently linked, the ends of the chains approaching from the two sides would be separated by the relatively large van der Waals distance, which is more than twice the bond distance between covalently linked carbon atoms. (This fact has been previously used in the calculation of micellar volumes in Chapter 6.)

In the direction parallel to the bilayer surface only one reflection at 4.2 Å was observed, corresponding, as previously stated, to the separation between closely packed extended hydrocarbon chains. This distance did not change upon the addition of water, and the overall conclusion is that the bilayer at the temperature of measurement (23°) has a core consisting of extended hydrocarbon chains essentially perpendicular to the bilayer surface, in a regular ordered array.

Similar results were obtained with stacked bilayers consisting of egg yolk phosphatidylcholine, with the important difference that the thickness of the bilayer was found to be smaller and the surface area per head group larger: about 60 $Å^2$ per head group or 30 $Å^2$ per hydrocarbon chain. This is larger than the head group area of 21 $Å^2$ for close-packed chains (p. 55) and indicates that the hydrocarbon chains cannot on the average be fully extended in this case, as indeed would not be possible in a mixture containing unsaturated hydrocarbon chains. In addition, the 4.2 Å reflection in the bilayer plane was replaced by a diffuse arc at 4.6 Å, showing not only a greater average separation between hydrocarbon chains, but also some variation from the 90° angle relative to the surface. The hydrophobic core in this case is clearly disordered, and the hydrocarbon chains must be undergoing considerable motion even though the bilayers themselves are in fixed positions. However, the trough in electron density at the center of the bilayer is still present, and the change in bilayer dimensions is not large. Substantial orientation of the chains and localization of CH_3 groups near the center evidently persists. It may be noted that increased hydration and separation between adjacent bilayers leads to slightly increased disorientation of the chains, delocalization of the ends, and slight shrinkage of the core thickness. This presumably results from the greater freedom of motion of the head groups.

The conclusions from X-ray diffraction studies have been confirmed by use of neutron diffraction (Worcester, 1976). Because the deuterium nucleus has a much higher scattering amplitude for neutrons than the hydrogen nucleus, the method is particularly valuable for determining the location of hydrogen atoms by comparison of scattering profiles obtained from natural and deuterated specimens. Substitution of D_2O for H_2O in hydrated ordered stacks of bilayers demonstrates unambiguously that the hydrocarbon core of the bilayer is essentially devoid of water.

True single crystals with three-dimensional order have been obtained for dilauroyl phosphatidylethanolamine, and the precise molecular structure has been determined by X-ray diffraction (Hitchcock et al., 1974). The crystals were grown from solutions of the lipid in glacial acetic acid, and the structure observed is thus not necessarily relevant to the structure in lipid bilayers in an aqueous system. Noncrystallographic data to be discussed in the following chapter indicate, however, that there is in fact close structural similarity between the molecules in the two states. The result of most general interest is the demonstration that the zwitterionic ethanolamine moiety of the lipid head group is oriented *parallel* to the plane of the bilayer, a result which other data show to be also applicable to the choline head group in diacyl phosphatidylcholines.

ARTIFICIAL MEMBRANES FOR PHYSIOLOGICAL STUDIES

For physiological studies (electrochemical properties, diffusion experiments, etc.) artificial membranes of large surface area, consisting of a single lipid bilayer, bounded on both sides by aqueous solutions, have proved to be extremely useful. Such membranes can be prepared by a procedure first developed by Mueller et al. (1962), which involves extension and drainage of a layer of lipid in an organic solvent, such as decane, until the two monolayers at the water–organic solvent interface coalesce. A scholarly review on the formation and properties of membranes of this kind has been written by Fettiplace et al. (1975). The membranes generally retain some of the solvent in which the lipid was originally dissolved (Henn and Thompson, 1968), so that they cannot be considered structurally identical to a pure phospholipid bilayer.

CHOLESTEROL AND ITS INCORPORATION IN PHOSPHOLIPID BILAYERS

Cholesterol is easily crystallized, either in the anhydrous state or as the monohydrate, and the molecular structure in both forms has been determined (Craven, 1976; Shieh et al., 1977). In the absence of other amphipiles its solubility in water is extremely low. It has been reported that micellelike aggregates with a broad size distribution are formed before the solubility limit is reached (Haberland and Reynolds, 1973), but recent data have raised a question about this and further investigation is needed. When other amphiphiles are present, however, mixed micelles with cholesterol may be formed. In particular, cholesterol can enter into phospholipid bilayers (even in their vesicular form) to a level of one molecule of cholesterol per molecule of phospholipid. Since the question of why cholesterol is present in membranes at all is still unsolved, mixed phospholipid-cholesterol bilayers have received much attention, in the hope that structural studies can provide a clue to the biological function.

X-ray diffraction studies of phospholipid-cholesterol bilayers were first reported by Small and Bourges (1966). In the most recent work by this technique (Levine and Wilkins, 1971; Franks, 1976) oriented hydrated bilayers of egg yolk phosphatidylcholine were used, containing 0.67 or 1.0 molecules of cholesterol per molecule of phospholipid. The resulting electron density profile was found to be qualitatively similar to that of Fig. 11-2, with somewhat expanded dimensions. All of the studies show that the cholesterol molecule is deeply embedded in the hydrocarbon core of the bilayer. A refinement of its position has been obtained by use of neutron-diffraction (Worcester and Franks, 1976): the change in the apparent density profile induced by deuterium labeling of the carbon atom adjacent to the OH group showed that the OH group must be located very close to the surface of the bilayer core, next to the ester links by which the fatty acyl chains are attached to the phospholipid head group. Brockerhoff (1974) has suggested that the OH group is hydrogen-bonded to the carbonyl group in this position and there is evidence for this from the effect of cholesterol on the ^{31}P-nmr spectrum of phospholipids (Yeagle et al., 1975). This would suggest that the incorporation of cholesterol into a phospholipid bilayer is not an example of simple mixed micelle formation driven solely by the hydrophobic effect: the hydrogen bond to the carbonyl group may be necessary to make location of cholesterol in the mixed bilayer thermodynamically favored over self-association. This would account for the inability to form mixed systems with more than a 1 : 1 ratio of cholesterol to phospholipid. The existence of a stoichiometric (1 : 1) compound between cholesterol and lysophosphatidylcholine (Rand et al., 1975) provides additional evidence for a specific mode of association.

It may be noted that a number of studies have shown that a critical point in the properties of phospholipid bilayers is reached when the molar ratio of phospholipid to cholesterol is 2:1 rather than 1:1. For example, the properties of small bilayer-bounded vesicles change only slowly up to this point, whereas a much more abrupt change occurs when the cholesterol content is further increased (Newman and Huang, 1975). This may reflect a purely geometrical phenomenon, unrelated to the mode of intermolecular association: the constituent molecules of a mixed bilayer containing up to 33 mol % cholesterol can be arranged in such a way that each cholesterol steroid ring is completely surrounded by phospholipid hydrocarbon chains, but this is no longer possible when the cholsterol content is higher (Engelman and Rothman, 1972).

BILE SALTS

The bile salts are important biological amphiphiles as well as useful laboratory reagents. Their molecular structure is not simple: the steroid ring is the major part of the hydrophobic moiety and the carboxyl group on an aliphatic chain at one end may be considered to be the "head group." However, there are also OH groups attached to the steroid ring, which make it impossible to form ordinary micelles of the type discussed in Chapter 6. Micellelike aggregates are formed, but hydrogen bonds between OH groups play an important role in their formation. Discussion of the extensive experimental studies of bile salt micelles and proposed structures for them are contained in an authoritative review by Small (1971).

MIXED MICELLES

According to the principles set forth in Chapter 9, any hydrophobic or amphiphilic molecule should ordinarily be able to enter into phospholipid bilayers, and the retention of organic solvent in artificial membranes (see above) is an example of this. If the added substance is an amphiphile with a single hydrocarbon chain (which category includes lysophospholipids), one would expect the mixture to retain its bilayer character only at low levels of the additive; progressive disruption of the bilayer and eventual formation of small micelles containing only small amounts of lipid should occur as the proportion of single-chain amphiphile is increased. This rather general prediction is borne out by everyday laboratory experience: the dispersion of phospholipids with detergents is a common experimental procedure. The equilibrium in these systems should not depend on the order of addition of the reagents, and we have pointed out above that one way of forming bilayer-bounded vesicles is by removal of detergent from mixed small lipid-detergent micelles.

The general principles of mixed micelle formation apply of course only when both pure components form micelles with liquid hydrocarbon cores. Thus bilayers with an ordered core structure, such as can be formed from pure phospholipids with identical saturated acyl chains, would resist incorporation of other amphiphiles. Cholesterol, too, is technically an exception to the general rule if, as suggested above, mixed micelle formation could not occur by nonspecific hydrophobic forces alone. The reason in this case lies in the crystalline state of the cholesterol, which is stabilized by specific intermolecular forces.

LIPID MONOLAYERS

Phospholipid monolayers at an air–water interface resemble monolayers formed by simpler amphiphiles, as shown in Fig. 10-2, with one important difference: because there are two hydrocarbon chains per head group, the head groups cannot approach as close to each other as in monolayers formed by amphiphiles with a single alkyl chain. Thus the minimum area per head group at high pressures is about 40 $Å^2$ instead of 20 $Å^2$. In the compression of liquidlike films, interactions between head groups are therefore less important than the packing ability of the hydrocarbon chains, as is seen for example from the work of van Deenen et al. (1962), who obtained quite similar pressure-area curves at the interface between air and 0.14 *M* phosphate buffer for the distearoyl derivatives of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidic acid, even though the last two have negatively charged head groups. On the other hand, phospholipids containing one unsaturated and one saturated alkyl chain formed considerably more expanded films.

At an interface between an aqueous solution and liquid hydrocarbon, somewhat surprisingly, continuous compression curves like those for single-chain amphiphiles in Fig. 10-2 are not always obtained. If the phospholipid is one that would form ordered paracrystalline arrays in a pure bilayer or at an air–water interface, an ordered monolayer phase is formed at moderate pressures at a hydrocarbon–water interface as well, as illustrated in Fig. 11-3, even though in this case this requires segregation (phase separation) of the lipid hydrocarbon chains from those of the solvent. This is clearly a two-dimensional analog of the problem mentioned above in connection with the formation of mixed lipid micelles, that is, the tendency for two components of a mixture to resist mutual solubility if one of them can form an ordered phase.

An important observation that has been made in a number of laboratories is that cholesterol does not form liquidlike monolayers at an air–water interface, but condenses at quite low surface pressures to a solidlike film with an area of about 39 $Å^2$ per molecule (Adam, 1941, p. 49; Pethica, 1969). This result is consistent with the behavior of cholesterol in aqueous solution. On the other hand, cholesterol enters as readily into phospholipid monolayers as it does into bilayers. When it does so, it exerts a "condensing effect," which means that the area of a mixed phospholipid–cholesterol monolayer at any pressure is smaller than would be predicted on the basis of additivity of the areas of the two components in the mixture (see review by Pethica, 1969). Presumably phospholipid and cholesterol interact in the same way in a monolayer as in a bilayer, and the decrease in area results from the intimate association

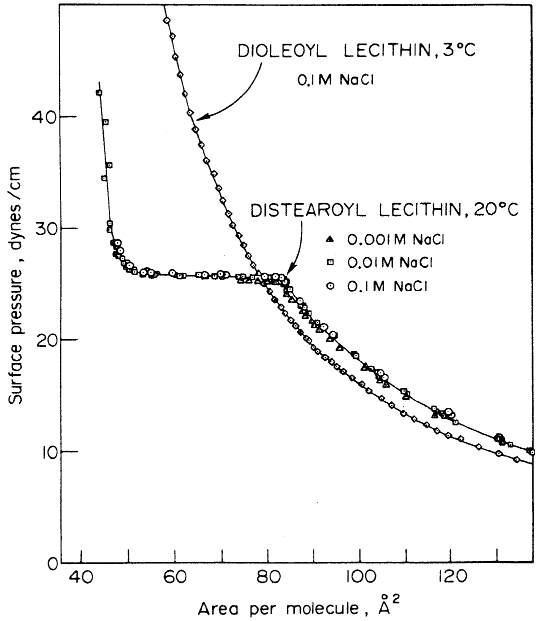

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Fig. 11-3. Pressure-area curves for two diacyl phospholipids at the interface between heptane and aqueous NaCl solutions (0.001 to 0.10 *M*, as indicated). The plateau observed for the distearoyl derivative, indicative of phase separation, disappears as the temperature for the order–disorder transition of pure distearoyl phosphatidylcholine is approached. From Yue et al. (1976).

between the two molecules that was postulated to occur in mixed bilayers.

It may be noted in conclusion that the methyl groups at the center of a phospholipid bilayer represent the region of lowest density (Fig. 11-2) and weakest intermolecular contacts. It is therefore not inappropriate to consider a bilayer as being nearly equivalent to two apposed monolayers. The fact that bilayer-bounded vesicles can be formed with very different numbers of molecules in the two halves (p. 115) is consistent with this, as are the structural similarities between monolayers and bilayers cited in this section. Additional evidence from phase transititions will be seen in the following chapter.

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TWELVE

MOTILITY AND ORDER

It was noted in Chapter 6 that long-chain unbranched saturated hydrocarbons form crystalline phases with melting points near or above room temperature, whereas the melting points of unsaturated hydrocarbons are much lower. X-ray diffraction data presented in the preceding chapter indicated that a somewhat analogous situation exists in the hydrophobic core of bilayer structures formed by biological lipids containing two hydrocarbon chains per molecule: in a pure lipid with two saturated hydrocarbon chains the chains appear to be in an ordered array at room temperature, whereas they are disordered in bilayers formed by lipid mixtures containing both saturated and unsaturated hydrocarbon chains. In the present chapter additional information obtainable by other methods will be examined to refine our picture of the state of the bilayer core. In addition, measurements of the motility of individual lipid molecules in and out of the bilayer and within it will be discussed.

FLUIDITY OF THE BILAYER INTERIOR

The methods described in Chapter 6 for study of the hydrocarbon core of micellar aggregates formed by simple amphiphiles have been applied extensively to phospholipid bilayers. At temperatures above the transition from ordered to disordered hydrocarbon chains, the general conclusions reached for simpler amphiphiles apply to lipid bilayers as well. For example, the solubility of small alkane molecules in the bilayer core is essentially the same as the solubility in small micelles (Stone, 1975). Raman spectroscopy (Gaber and Peticolas, 1977) can distinguish between *trans* and *gauche* configurations of hydrocarbon chain segments, and the existence of *gauche* conformations as the basis for chain disorder (Fig. 6-1) has been directly confirmed. Spectroscopic relaxation methods have been used to show that the phospholipid hydrocarbon chains are in a fluid state. One of the earliest measurements of this kind (Chapman and Penkett, 1966) showed that proton nmr spectra in bilayers are quite similar to spectra observed for phospholipids in solution in an organic solvent such as $CHCl_3$, and subsequent more sophisticated analyses have shown that the hydrocarbon chains are in rapid anisotropic motion about an axis roughly perpendicular to the bilayer surface (see review by Lee, 1975). As with simpler amphiphiles, the chain segments nearest to the head group are greatly restricted in their freedom of motion, and those near the center of the bilayer core are much freer.

As the methods for examining hydrocarbon chain motion have become more refined, some differences between phospholipid bilayers and micellar structures of simple amphiphiles have become apparent. The most important difference was demonstrated by deuterium magnetic resonance (dmr) studies of selectively deuterated dipalmitoyl phosphatidylcholine, made at 50°C, where the bilayers are well above the order–disorder transition temperature (Seelig and Seelig, 1975). The dmr spectrum obtained when the first CH_2 group of the R_1 chain (formula I, p. 107) is deuterated is not the same as the spectrum obtained when the corresponding group in R_2 is deuterated. The difference between the two spectra suggests that the first segment of the R_2 chain is oriented parallel to the bilayer surface and that the chain has to bend sharply beyond this point to assume an orientation perpendicular to the surface. A consequence of this is that the two chains remain slightly out of alignment throughout the bilayer, and that, if the two chains have the same overall length, the R_2 chain cannot penetrate quite as deeply into the bilayer. These features of the molecular conformation are exactly the same as those deduced from determination of the crystal structure of dilauroyl phosphatidylethanolamine, which was mentioned in the preceding chapter.

These and related results have been reviewed by Seelig (1977). The same review discusses differences between conclusions based on spin label esr measurements and the newer dmr data. The esr measurements (Hubbell and McConnell, 1968, 1971; McFarland and McConnell, 1971; Jost et al., 1971) were the first to provide detailed information on this subject and to establish the general features of bilayer organization, but it now appears that they were influenced to some extent by local perturbation of the hydrocarbon chains by the presence of the nitroxide spin label group on the probe molecules used in the studies.

Most naturally occurring phospholipid molecules contain an unsaturated chain in the R_2 position. One synthetic molecule of this type, 1-palmitoyl-2-oleoyl phosphatidylcholine has been studied by dmr. Hydrocarbon chains in bilayers formed by this lipid are more disordered than in bilayers of dipalmitoyl phosphatidylcholine studied at the same temperature, undoubtedly because unsaturated and saturated chains cannot have exactly parallel alignment. If one compensates for this by studying the two lipids at different temperatures, such that each is at the same number of degrees above its order-transition temperature, an ordering effect of the double bond in the R_2 chain can be observed, that is, the local rigidity of this bond reduces the motional freedom of CH_2 groups in adjacent chains (Seelig, 1977).

Spectroscopic relaxation methods have also been used to obtain information about the motion of polar head groups in bilayers. Different head groups exhibit very different behavior. Both proton magnetic resonance (Michaelson et al., 1974) and dmr (reviewed by Seelig, 1977) show that the head group of phosphatidylethanolamines is much more restricted in its motion than the head group of phosphatidylcholines, undoubtedly a reflection of the larger size of the $N(CH_3)_3^+$ group relative to NH_3^+, which makes the former harder to fit into a tightly packed array. Hydrogen bonds between NH_3^+ and the phosphate group of a neighboring molecule may also contribute to rigidity in phosphatidylethanolamines. Below the phase transition temperature the phosphatidylethanolamine head groups are completely immobilized, but choline head groups retain freedom of motion even when the hydrocarbon chains are in the ordered state. The head group appears to lie parallel to the plane of the bilayer and its motion appears to be in that plane, a result consistent with X-ray and neutron diffraction data. The motion of the choline head group may not consist of a continuous rotation with respect to the rest of the molecule, but may consist, instead, of an oscillation between two alternative enantiomeric configurations. The crystal structure determined for L-α-glycerophosphorylcholine (i.e., a phospholipid analog lacking the two acyl chains) in fact has a unit cell containing two molecules in appropriate enantiomeric conformations (Abrahamsson and Pascher, 1966).

PHASE TRANSITIONS

Pure anhydrous phospholipids are solids with very high melting points. The melting points are unrelated to hydrocarbon chain length or degree of unsaturation. This in itself suggests that the hydrocarbon chains are already in a liquidlike state in the solid at the melting point, and that the melting process primarily reflects the disruption of intermolecular bonds between head groups. Support for this conclusion is provided by the observation of a major phase transition within the solid state at temperatures below the true melting point. This phase transition occurs at the same temperature as the transition of the interchain X-ray spacing from 4.2 to 4.6 Å in those instances where X-ray diffraction measurements as a function of temperature have been made, and is generally taken to reflect an order–disorder transition of the hydrocarbon chains within the solid. Similar phase transitions, but at temperatures below those at which they occur in the anhydrous solid, can be observed in aqueous phospholipid dispersions (multilayer structures as in Fig. 11-1*a*) and in small single-walled vesicles and in monolayers. Studies of such phase transitions have made significant contributions to our understanding of phospholipid bilayers. There is a general review, emphasizing theoretical aspects, by Lee (1977).

The most precise way to observe the transition is by differential scanning calorimetry (Ladbrooke and Chapman, 1969; Chapman et al., 1974). In this method a profile of heat absorption versus temperature is obtained. In the absence of phase transitions a monotonic line is obtained, the slope of which measures the heat capacity of the system. Phase transitions are characterized by additional heat absorption over narrow temperature ranges: both the transition temperature and the enthalpy change in the process are directly determinable. Calorimetry by itself is of course mechanistically uninformative, and the spectroscopic and other procedures already cited have to be relied on to identify the molecular processes responsible for the absorption of heat.

Examples of data obtained by calorimetry are given in Table 12-1. They show at once that the observed process is not directly comparable to the melting of hydrocarbon crystals, for which data were given in Table 6-2. The observed transition temperatures are higher than the melting points of hydrocarbons of comparable chain lengths. The transition temperatures differ between phospholipids with different head groups, and they are much higher in anhydrous crystals than when the lipid is hydrated, a clear indication that head group interactions play an important role. Direct evidence that a loosening of head group organization accompanies the order–disorder transition of the hydrocarbon chains was first given by Träuble (1971). He also showed that the transition is highly cooperative, as expected, and more exact information on this is provided by examination of the shape of the heat absorption profiles in calorimetry experiments (Hinz and Sturtevant, 1972; Mabrey and Sturtevant, 1976).

Table 12-1 also shows that the ΔH values are lower than the ΔH values for the melting of pure hydrocarbons. The same is true for the entropy change in the process (Phillips et al., 1969) which is only about half as large as the ΔS values given in Table 6-2 for hydrocarbons with an odd

Table 12-1. Calorimetric Data for the Transition from Ordered to Disordered Hydrocarbon Chains in Diacyl Phospholipids[a]

Phospholipid[b]	Transition Temperature (°C)	ΔH(kcal/mol) per Mol Lipid	ΔH(kcal/mol) per Hydrocarbon Chain
	Anhydrous Crystalline State		
diC_{22} phosphatidylcholine	120	16.8	8.4
diC_{18} phosphatidylcholine	115	13.8	6.9
	Hydrated Multilayers[c]		
diC_{22} phosphatidylcholine	75	14.9	7.4
diC_{18} phosphatidylcholine	54.9	10.6	5.3
diC_{16} phosphatidylcholine	41.4	8.7	4.4
diC_{14} phosphatidylcholine	23.9	5.4	2.7
di$C_{18:1}$ phosphatidylcholine	−22	7.6	3.8
diC_{16} phosphatidylethanolamine	60	8.5	4.2
diC_{14} phosphatidylethanolamine	49.5	5.8	2.9
diC_{16} phosphatidylserine (low pH)[d]	72	9	4.5
(high pH)	55	3	1.5
	Small Single-Walled Vesicles		
diC_{16} phosphatidylcholine	37	—	—

[a] Data from Phillips et al. (1969), Hinz and Sturtevant (1972), Mabrey and Sturtevant (1976), van Dijck et al. (1976), MacDonald et al. (1976), and Suurkusk et al. (1976).

[b] Each molecule contains two identical chains. The numerical subscript indicates the number of carbon atoms of the acyl chain (18 : 1 = oleoyl), including that of the ester group. The number of carbon atoms in the hydrocarbon chain is one less than this.

[c] As illustrated by Fig. 11-1*a*.

[d] The phosphatidylserine head group is zwitterionic at low pH, but acquires a net negative charge when the serine COOH group becomes ionized, the exact pH of the addition depending on the added electrolyte. Charge effects on phase transitions are discussed theoretically by Träuble (1977).

number of carbon atoms, and even smaller in relation to hydrocarbons with an even number of carbon atoms. The change in heat capacity is also much smaller than for the melting of pure hydrocarbons (Hinz and Sturtevant, 1972). These observations all show that the hydrocarbon chains in the "melted" bilayer core are not as disordered as they are in a pure liquid hydrocarbon. Nevertheless, the effects of hydrocarbon chain length and of unsaturation are qualitatively similar for the two processes. All the results are consistent with the conclusions reached in Chapter 6 about the restricted motion of hydrocarbon chains in micellar aggregates, and as discussed above for bilayers in particular.

It should be mentioned that calorimetric studies show that there is a "pretransition" in phosphatidylcholines, but not in phosphatidylethanolamines, which occurs a few degrees below the temperature for the main order–disorder transition. ΔH for the process is small, and it is thought to be associated with a subtle modification in the arrangement of choline head groups (Janiak et al., 1976).

The order–disorder transition can be observed in small single-walled vesicles as well as in more extended multilamellar structures. Because of the large curvature of the bilayers in these vesicles, the transition occurs at a somewhat lower temperature (Table 12-1) and the vesicles become unstable if the temperature is lowered to the point where all of the lipid is converted to the ordered state (Suurkusk et al., 1976). The transition can also be observed in lipid monolayers at the interface between liquid hydrocarbon and an aqueous solution. As mentioned in the previous chapter, separation of an ordered phospholipid phase is observed when such monolayers are compressed. As the temperature is increased there is a corresponding rise in the pressure required for phase separation, and, at a temperature close to the transition temperature observed in bilayers, phase separation can no longer be observed (Yue et al., 1976). This is further evidence for the weakness of direct interactions between the two halves of a bilayer (p. 125).

Spectroscopic methods have also been used to examine these phase transitions. When the spectroscopic method reflects the behavior of the hydrocarbon chains directly (e.g., Raman spectroscopy, Gaber and Peticolas, 1977), the observed transition temperature is the same as observed by calorimetry. Transition temperatures measured by use of a spin-label or fluorescent probe tend to fall slightly below those measured by direct observation (e.g., Lentz et al., 1977). The change reflects the fact that the probe is soluble only in a liquidlike phase and thus lowers the transition temperature according to the same thermodynamic principle that causes lowering of the melting point of an ordinary crystal when an additive soluble in the liquid is present. Many substances added to a lipid-water system tend to be more soluble in the disordered phase and thus lower the transition temperature in the same way. A notable exception is provided by the addition of palmitic acid to dipalmitoyl phosphatidylcholine at a 2:1 molar ratio (Mabrey and Sturtevant, 1977). The transition temperature is raised from 41 to 61°, indicating formation of a very stable ordered phase by the mixture.

Phase transitions in mixtures of different lipids occur over a much broader range of temperature than for pure lipids (e.g., Phillips et al., 1972; Shimshick and McConnell, 1973). The components of the mixture are usually mutually not very soluble in the ordered phase, so that the higher-melting component may separate out as a pure crystalline phase in equilibrium with a liquid phase enriched in the lower-melting component or components. (The appearance of an ordered lipid phase in monolayer compression studies at an aqueous–hydrocarbon interface, which was mentioned earlier, is an example of the same basic phenomenon.) As the temperature in a mixed lipid system is reduced below the normal transition temperatures of lower-melting components, two or more distinct ordered phases can exist in equilibrium with the fluid phase. It can also happen that the components of a lipid mixture are incompletely miscible in the liquid state, so that lateral separation into two fluid phases of different composition can occur. Phase separation can sometimes be induced by addition of another substance: for example, Ca^{2+} binds to the head group of phosphatidylserine and causes phase separation in a mixture of phosphatidylserine with phosphatidylcholine (Ohnishi and Ito, 1974). It is important to note that the continuity of the bilayer form is maintained in all phase separations of this kind: the separate phases exist as small clusters within an otherwise intact overall morphology.

MOBILITY OF LIPID MOLECULES

The ability to define and measure a unique monomer concentration in equilibrium with phospholipid dispersions implies the existence of a dynamic equilibrium between lipid molecules in a bilayer and in the solution with which it is in contact. However, the fact that the monomer concentration is very small, of order $10^{-10}M$ for typical lipids (Table 11-2), means that the rate of exchange between bilayer and surroundings must be slow. No direct measurements have been made, but Kornberg and McConnell (1971a) have measured the rate of exchange of spin-labeled phosphatidylcholine between small phosphatidylcholine vesicles which, under their experimental conditions, was very slow indeed (average exchange time of the order of 24 hr). Even this slow exchange probably occurred in their experiments by direct transfer between colliding vesicles, and not by dissociation of a labeled molecule from one vesicle, followed by diffusion and incorporation into a new vesicle.

Another slow process is the interchange of lipid molecules between one side of the bilayer and the other, the popular name for which is "flip-flop." As already noted, phospholipid bilayers have very low permeability for ions of all kinds, even in small vesicles, and interchange clearly cannot occur without passage of the ionic or zwitterionic head group of the lipid molecule through the hydrophobic core. Measurements of the rate of the process were first made by Kornberg and McConnell (1971b). They used a phosphatidylcholine with a nitroxide spin label attached to the choline head group and made use of the fact that the nitroxide group is readily reduced to an inert product by ascorbic acid. Egg phosphatidylcholine vesicles similar to those described on page 114 were prepared, bounded by a single bilayer, and containing an internal solvent-filled cavity. A small percentage of spin-labeled molecules was added to the lipid from which the vesicles were made, and was found to distribute itself more or less randomly between the outer and inner surface of the bilayer: 65% of the initial esr intensity could be removed by reduction with ascorbate, which had been shown previously not to enter the internal cavity of the vesicle. The rate of interchange between the two bilayer surfaces was then measured by following the appearance of more reducible label as a function of time; this was taken to represent labeled molecules that had crossed from the inner to the outer surface. The half-time of the process was found to be 6.5 hr at 30°, corresponding to a probability of 0.07/hr for the passage of a given molecule from the internal to the external surface. More recent experiments avoiding the use of a probe molecule indicate an even slower rate for trans-membrane interchange. For example, Rothman and Dawidowicz (1975) employed a specific exchange protein that binds phosphatidylcholine and catalyzes rapid exchange between the bound lipid and phosphatidylcholine in bilayer membranes. As expected, about two-thirds of the lipid in phosphatidylcholine vesicles was found to be accessible for immediate exchange. However, the appearance of additional exchangeable lipid (from the inside half of the bilayer) was not observed to take place at a measurable rate, leading to the conclusion that the half-time for exchange must be greater than 11 days at 37°C. Shaw et al. (1977) were able to measure the rate of exchange on the basis of head group accessibility to a paramagnetic shift reagent, their estimated half-time being 16 to 69 days at 23°C.

In contrast to the two preceding kinds of motion, lateral diffusion of phospholipid molecules in the plane of the bilayer is extremely rapid, so that lipid molecules with labeled head groups introduced at high concentration at one point of a bilayer vesicle, for example, would become uniformly distributed over the entire surface in less than a second. The diffusion coefficient has been evaluated by Devaux and McConnell (1972), again by use of nitroxide labeled phosphatidylcholine. The broadening of the resonance line in the esr spectrum by spin–spin interaction with increasing concentration of the label was used to establish an empirical measure of concentration. Ordered multilayers (page 118) of lipid containing a high concentration of labeled molecules were then laid down[1] and fused with multilayers containing no label. The rate of diffusion of the label into the unlabeled areas was then measured. The diffusion coefficient was found to be $1.8 \pm 0.6 \times 10^{-8}$ cm^2/sec at 25°, corresponding to an average displacement of about 10^{-4} cm/sec as a result of the random motion of a single molecule. Diffusion rates based on the enhancement of nuclear relaxation rates by the presence of a small proportion of spin-labeled molecules are consistent with this value. Measurements of this kind indicate that diffusion in small single-walled vesicles may be significantly faster than in multilayers (Kornberg and McConnell, 1971a; Brûlet and McConnell, 1975). Lateral diffusion rates in planar bilayers (artificial membranes, p. 121) have been measured directly by use of fluorescence correlation spectroscopy (Fahey et al., 1977). The diffusion coefficients observed were found to be severalfold larger than those obtained from spectroscopic relaxation methods.[2]

EFFECT OF CHOLESTEROL

Rothman and Engelman (1972) suggested, primarily on the basis of model building, that the rigid steroid ring of cholesterol should be rather immobile and that incorporation of cholesterol in a phospholipid bilayer should further decrease freedom of motion of the already constrained phospholipid hydrocarbon chain segments which are close to the polar head groups. On the other hand, fluidity should be increased near the central portion of the bilayer, where the branched paraffin chain of the molecule is located. Proton nmr measurements, looking at cholesterol protons in a deuterated phospholipid bilayer, provide experimental evidence in support of this: they show that the isopropyl moiety at the end of the paraffin chain has essentially unrestricted motion, whereas motion of the steroid ring is greatly restricted (Kroon et al., 1975). The rate of trans-bilayer interchange for cholesterol molecules in a phospholipid bilayer is probably as slow as it is for the phospholipid molecules (Poznansky and Lange, 1976).

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[1] The bilayers in most of these experiments were prepared from dihydrosterculoyl phosphatidylcholine, a synthetic lipid analogous to dioleoyl phosphatidylcholine, but containing a cyclopropane ring in place of the double bond. This was done because the high concentration of the free radical label required for this experiment leads to slow peroxidation of double bonds when they are present. The hydrosterculoyl chain is sterically very similar to the oleoyl chain, as the cyclopropane ring introduces a fixed 120° bond angle into the structure just as a double bond does.

[2] It is important to note that the coefficient for linear diffusion in a two-dimensional fluid is not entirely analogous to the diffusion coefficient in a three-dimensional fluid (Saffman and Delbrück, 1975).

When attempts are made to measure the effect of cholesterol on the order–disorder phase transition of phospholipids by calorimetry, the heat absorption associated with the process is seen to vanish when the cholesterol content reaches 1 mol for every 2 mols of phospholipid (Hinz and Sturtevant, 1972b). Raman spectral data (Lippert and Peticolas, 1971) show, however, that the *gauche* to *trans* configurational change is not abolished, but that it occurs noncooperatively over a very broad temperature range. These results are consistent with the structural arrangement of phospholipid and cholesterol molecules in the mixed bilayer that was suggested in the preceding chapter.

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THIRTEEN

PROTEINS

HYDROPHOBIC SIDE CHAINS AND CONFORMATIONAL CHANGE

Proteins are amphiphilic molecules. They contain amino acids with hydrocarbon and other hydrophobic side chains, and also amino acids with ionic and uncharged polar side chains. The polypeptide backbone itself contains the strongly hydrophilic peptide group, but its effect can be nullified by formation of interpeptide hydrogen bonds, such as exist in the α-helical and parallel extended chain β-structures of the polypeptide chain (Pauling et al., 1951). The network of hydrogen bonds in such structures appears to have comparable stability with hydrogen bonds between the peptide groups and water, and polypeptide *backbones* that have adopted this type of conformation no longer retain a marked preference for being in an aqueous environment.

Measurements of the solubilities of amino acids and related substances, in water and in organic solvents such as ethanol, have been used to assess the contributions of the side chains to the free energy of transfer from water to the organic solvent (Cohn and Edsall, 1943; Nozaki and Tanford, 1971). The numerical values of these parameters are shown in Table 13-1. For the side chains containing only hydrocarbon they are smaller than one would expect on the basis of the $\mu_{HC}^{\circ} - \mu_W^{\circ}$ values given in Chapters 2 and 3; for example, the difference between leucine and glycine corresponds to the insertion of four CH_2 groups, yielding −650 cal/mol per CH_2 group, as compared to about −850 cal/mol for $\mu_{HC}^{\circ} - \mu_W^{\circ}$. Part of this discrepancy arises here from the use of ethanol or dioxane as the organic solvent rather than pure hydrocarbon (cf. Table 2-2), but a second contributing

Table 13-1. Hydrophobicity of Protein Side Chains[a,b]

Side Chain	$\mu^\circ_{\text{org}} - \mu^\circ_W$ (cal/mol)
Tryptophan	−3400
Norleucine[c]	−2600
Phenylalanine	−2500
Tyrosine	−2300
Leucine	−1800
Valine	−1500
Methionine	−1300
Alanine	− 500

[a] The figures given represent the *additional* free energy of transfer from water to an organic solvent that is generated when the side chain listed is substituted for the hydrogen atom of a glycyl residue. Most of the data are derived from experiments in which the organic solvent was ethanol or dioxane at 25°C (from Nozaki and Tanford, 1971).

[b] The principal hydrophobic side chains include, in addition to those listed, isoleucine, proline, and cystine. Experimental values of $\mu^\circ_{\text{org}} - \mu^\circ_W$ are not available for them. Portions of some hydrophilic side chains are hydrophobic, notably the $-(CH_2)_4-$ moiety of lysine, which is likely to be characterized by a $\mu^\circ_{\text{org}} - \mu^\circ_W$ value of −1.0 to −1.5 kcal/mol. This free energy decrease can of course be realized only if the terminal $-NH_3^+$ group remains in contact with water at the same time as the $-(CH_2)_4-$ moiety is removed from such contact.

[c] Norleucine is not a naturally occurring side chain and is included here as a reference compound. Essentially identical results have been obtained with ethanol, butanol, and acetone as the organic solvent.

factor is probably the fact that the side chains are short and attached to a CH group located between two polar groups. As previously stated (p. 14), a CH_2 group attached directly to a polar group (in a linear molecule) is likely to be much less hydrophobic than a CH_2 group far removed from a polar group of the same molecule. The same factor is likely to apply to a lesser degree here. (Note that the figure for alanine in Table 13-1, corresponding to insertion of only the proximal CH_2 group, is only −500 cal/mol.) In any event, there is no question that the hydrocarbon side chains are hydrophobic, and that the magnitude of the unfavorable free energy of forming an interface with water increases with the size of the side chain: the aromatic side chains are larger than aliphatic ones and make the largest contributions.

In water-soluble proteins about 25 to 30% of the amino acid side chains are generally significantly hydrophobic, and 45 to 50% are typically ionic or contain uncharged hydrophilic side chains. The rest (in which we have included alanine from among the side chains listed in Table 13-1) have relatively little preference for being in or out of the aqueous environment. In the native conformation of such a protein, a substantial fraction of the hydrophobic side chains are typically buried in the interior of the molecule, the free energy gained thereby being a major factor in the stability of the native conformation (in water) relative to a more flexible conformation in which the hydrophobic side chains would be exposed to solvent (Tanford, 1962). The native conformation is, in fact, in dynamic equilibrium with other possible conformations, and reversible transitions to other conformations take place when the solvent medium is altered (Tanford, 1968). The transition from a disordered state to the native conformation thus has some resemblance to micelle formation, and, like micelle formation, it is a highly cooperative process: structures in which just a few hydrophobic side chains are removed from an aqueous environment are generally not stable.

The similarity between micelle formation and protein folding is, however, only superficial. The hydrophobic amino acids are not grouped together in the polypeptide chain, but are interspersed with amino acids having ionic or polar side chains, each individual protein polypeptide chain having a unique sequence. Moreover, because the side chains are so short, much of the polypeptide backbone to which they are attached has to be incorporated into hydrophobic domains that are formed in the interior of the native structure, and, as noted above, this requires the formation of a network of interpeptide hydrogen bonds in order to nullify the otherwise strong affinity of the peptide group for water. Polar uncharged side chains are under a similar constraint, and, if located in a portion of the polypeptide chain that is to be incorporated in a hydrophobic domain, must form hydrogen bonds to other polar groups if the free energy is to be minimized.

The most severe restriction of all applies to the ionic amino acid side chains of arginine, lysine, glutamic acid, and aspartic acid. These side chains cannot be removed from an aqueous environment without severe loss of free energy. As in the formation of ionic micelles, they must remain at the surface of the globular structure, and, indeed, virtually no exceptions to this principle have been found in proteins whose three-dimensional structure has been determined. Since the ionic side chains cannot be physically separated from neighboring hydrophobic amino acids, the limitations on possible stable structures are obvious, and polypeptide chains containing the normal proportions of the various kinds of amino acids, in an arbitrary sequence, may not be able to form stable globular structures at all. The amino acid sequences found in nature may represent a special group, selected by the evolutionary process because they are capable of forming structures in which hydrophobic amino acids can be segregated in internal domains without violating the requirements regarding location of ionic side chains and hydrogen bonding between peptide groups and other internal polar groups. Even in the stable structures, many hydrophobic side chains remain in at least partial contact with water, alanine especially so (as might be expected from the small value of $\mu^\circ_{\text{org}} - \mu^\circ_W$ in Table 13-1), and also proline (Lee and Richards, 1971).

One of the consequences of the foregoing restrictions is that the structure of native proteins must be quite rigid. The internal hydrophobic domains must be in a uniquely ordered state, quite different from the fluid interior of a micelle. Rotation of individual groups on side chains within a domain can occur, at a rate depending on how severely neighboring groups interfere with rotation (Gelin and Karplus, 1975), and nmr data have provided direct evidence to show, for example, that some, but not all, tyrosine phenolic rings undergo rapid rotational motion in the native state of one particular protein (Snyder et al., 1975; Wüthrich and Wagner, 1978). Interchange between adjacent whole side chains and other motions that occur freely in micellar aggregates cannot, however, normally take place. As a result of this, and of the incomplete avoidance of all contact with water, the free energy gained by removing hydrophobic side chains from water in formation of the native structure is actually much less than the free energies of transfer listed in Table 13-1 would predict. It is, in fact, found experimentally that native structures frequently have only marginal stability relative to disordered conformations or alternate structures (see below).

Much current research is devoted to a more precise analysis of the factors discussed here, to their interaction in determination of the optimal structure of the native protein, and to the sequence of events in the attainment of this structure. Some of this work is done with the goal of predicting the preferred native structure from knowledge of the amino acid sequence. Richards (1977) has written a concise review of the overall problem.

BINDING SITES FOR HYDROPHOBIC LIGANDS

Another consequence of the difficulty in the formation of a stable globular structure is that *complete* removal of hydrophobic side chains from contact with water is generally not possible. In most native proteins some hydrophobic groups remain exposed at the molecular surface or in crevices. If sufficiently large hydrophobic patches are formed, they may constitute binding sites for hydrocarbon or amphiphile molecules. The standard free energy of transfer of ligand molecules to such sites on the protein molecule ($\mu_P^\circ - \mu_W^\circ$) may be more negative than $\mu_{HC}^\circ - \mu_W^\circ$ for a hydrocarbon or $\mu_{\text{mic}}^\circ - \mu_W^\circ$ for an amphiphile because the process of binding may involve elimination of a hydrocarbon–water interface at the protein surface as well as that associated with the free ligand in the aqueous medium. Other factors exist, of course, that may diminish the affinity between protein and hydrophobic ligand. For example, the hydrocarbon–water interface may be only partially eliminated by binding to a surface site, or the bound ligand may be required to adopt an unfavorable conformation when it is bound. Another possibility is that energetically unfavorable local rearrangements of protein structure near the binding site may occur as part of the association process.

In the association of amphiphiles with hydrophobic binding sites of native proteins, the hydrophilic head group may play a significant role. An obvious example would be an ionic amphiphile. The electrostatic repulsion between head groups makes a significant positive contribution to $\mu_{\text{mic}}^\circ - \mu_W^\circ$, whereas electrostatic attraction between a head group and an ionic protein side chain with opposite charge could make a negative contribution to $\mu_P^\circ - \mu_W^\circ$.

The overall conclusion from these considerations is that native protein molecules may have hydrophobic sites suitable for association with hydrocarbons or amphiphiles, and that the free energy associated with binding to these sites is, in general, unpredictable and may be larger or smaller than the free energy associated with competing processes such as micelle formation.

CONFORMATIONAL CHANGE

The native conformation of a protein molecule possesses only marginal stability because it is highly constrained. Other conformational states, in which a much larger fraction of the hydrophobic side chains is exposed to the solvent than in the native state, are thus readily accessible (Tanford, 1968). One such state is the randomly coiled state, in which the protein polypeptide chains become flexible and all hydrophobic residues are exposed to the solvent. The free energy of transition to this state is small even in aqueous solution, typically about 100 cal/mol per amino acid residue (Tanford, 1970), because the creation of a large hydrocarbon–

water interface is largely compensated for by increased freedom of internal motion of the polypeptide chain.

Another denatured state which, like the randomly coiled state, may be accessible to most proteins is characterized by having a large fraction of the polypeptide chains in an α-helical conformation. It has never been carefully investigated, but it is likely to consist of several lengths of helical polypeptide chain, with flexible joints between them, and most of the hydrophobic side chains exposed to solvent. This state is commonly observed when proteins are dissolved in mixtures of water with alcohols and other organic solvents, in which exposure of hydrophobic residues is no longer attended by an unfavorable free energy change. The free energy of transition in a purely aqueous solution has not been determined, but it is likely to be higher than the free energy of transition to the randomly coiled state.

Protein molecules in such altered conformational states, with many exposed hydrophobic groups, are likely to be able to bind a large number of hydrophobic ligands per molecule. The free energy gained thereby (with $\mu_P^\circ - \mu_W^\circ$ intrinsically very negative) may greatly exceed the unfavorable free energy change accompanying the conformational change per se, and the transition to the altered conformational change would then be induced by the presence of the ligand. It is actually well known that detergents are good denaturing agents, and that they induce formation of a "helical" state that may be similar to the state attained by proteins in mixtures of water with organic solvents (Tanford, 1968; Jirgensons and Capetillo, 1970, Jirgensons, 1976).

Lesser conformational changes exposing a small number of hydrophobic side chains may occur in some proteins. Dissociation of an oligomeric protein to its subunits, if it takes place without appreciable conformational change within the individual subunits, would be an example. Minor readjustments of surface groups are likely to occur whenever a complex ligand binds to a site on a protein molecule. These would not be detected by any but the most refined methods for examining protein structure, and will not be considered as representing significant conformational change as this term is used here.

It is important to note that the gross disruption of the native conformation of proteins, including processes in which only a portion of the protein molecule is involved, are usually highly cooperative processes; they cannot occur in stepwise fashion, with exposure of one amino acid side chain at a time. This will be seen to have a significant effect on the binding of hydrophobic ligands to proteins that have to be denatured to make binding sites available.

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FOURTEEN

THE ASSOCIATION OF HYDROCARBONS AND AMPHIPHILES WITH COMMON SOLUBLE PROTEINS

As was pointed out in the preceding chapter, the existence of hydrophobic amino acid side chains on protein molecules suggests the possibility of interaction between proteins and small molecules containing hydrocarbon chains, such as hydrocarbons themselves, simple amphiphiles, and biological lipids. Interactions of this type presumably play a major role in the formation of functioning biological membranes, but, before we proceed to discuss membranes per se, it is worthwhile to examine available data on such interactions in simpler systems in which the protein is free in solution. This will be done in this and the following chapter.

INTERPRETATION OF EXPERIMENTAL BINDING ISOTHERMS

In nearly all examples in which we are interested the number of ligand molecules capable of binding to a single protein molecule is large, and the binding isotherms take the form of plots of the average number of bound ligands per protein molecule ($\bar{\nu}$) as a function of the concentration of *unbound* ligand molecules in the aqueous solution. There are several entirely distinct modes of association that can lead to high ligand/protein binding ratios, and to define them it is simplest to limit the initial discussion to situations where all unbound ligand exists in monomeric form, postponing until later in the chapter a consideration of the effects of self-association of the ligand. The concentration of free monomeric ligand will be expressed in mol fraction units; the symbols X_W and μ_W° defined by equation 2-1 will be used. Because binding usually occurs at very low ligand concentration, the activity coefficient f_w will be set equal to unity. Experimental data are usually in molar units (C_W), and the results are most often given in terms of the *total* unbound ligand concentration, which, in the case of amphiphiles, may include micelles as well as monomers. Throughout the discussion, the maximum possible value of $\bar{\nu}$ at saturation of the protein will be designated by the symbol n.

Discrete Binding Sites for Single Ligands

As noted above, a protein in its *native* state may have binding sites for hydrophobic ligands. If there are n such sites, and if the sites are identical and independent, equilibrium at each site is governed by the process, site (S) + ligand (L) $\rightleftharpoons$ SL, and the shape of the isotherm is exactly the same as it would be for binding to a large number of small molecules, each having only one site. The equation for the isotherm is

$$\bar{\nu}/(n - \bar{\nu}) = KX_W \qquad (14\text{-}1)$$

The isotherm is broad (as in portion *a* of Fig. 14-1): two log units in X_W or C_W are required to progress from 10% saturation of the sites to 90% saturation, that is, from $\bar{\nu} = 0.1\ n$ to $\bar{\nu} = 0.9\ n$.

The equilibrium constant K in equation 14-1 is the equilibrium constant for the reaction S + L $\rightleftharpoons$ SL, and (if the entire isotherm obeys the equation) can be determined experimentally from the midpoint of the isotherm, since $X_W = 1/K$ when $\bar{\nu} = n/2$. The value of K is related to the standard free energy of transfer ($\mu_P^\circ - \mu_W^\circ$) of the ligand to the protein site by the relation

$$-RT \ln K = \mu_P^\circ - \mu_W^\circ \qquad (14\text{-}2)$$

and the result will be in unitary units (p. 5) if mol fraction units of concentration are used. The factors influencing $\mu_P^\circ - \mu_W^\circ$ were discussed in the preceding chapter, and it was concluded that numerical values are not readily predictable.

If an experimental binding isotherm is even *broader* than equation 14-1 predicts, it means that a progressively smaller value of K must be used as X_W and $\bar{\nu}$ increase, and there are two possible interpretations. One is that the n binding sites are still independent, but they do not have identical affinity for the ligand. In that case the sites for higher affinity are filled at lower X_W, that is, the overall isotherm will be a superposition of individual isotherms, each obeying equation 14-1, but with a different K and thus a different midpoint. (In this situation the midpoint of the overall isotherm would yield an average value for K.) On the other hand, a broadening of the isotherm could also result from n identical binding sites if there is interaction between the sites, such that ligand binding at any one site interferes with binding at other sites (e.g., by steric or electrostatic repulsions). Additional experiments have to be done to distinguish between the possible mechanism for broadening; for example, broadening arising from electrostatic interaction would be expected to diminish by an increase in ionic strength.

Intrinsic Cooperativity

When a binding isotherm is steeper than equation 14-1 predicts, it means that there is an *increase* in binding affinity as more ligand is bound. Ligand binding of this kind is called *cooperative*.[1] The most extreme kind of cooperativity is obtained when the association with the protein does not involve sites for binding single ligands at all, but when there is instead a binding *region* that is intrinsically designed for binding a large number of ligands simultaneously. The reaction occurring would then be $P + nL \rightleftharpoons PL_n$, where P represents a native protein molecule, and, if intermediate forms such as PL, PL_2, and so on are not sufficiently stable to contribute to the equilibrium mixture at any ligand concentration, the binding isotherm would obey the equation

$$\bar{\nu}/(n - \bar{\nu}) = KX_W^n \qquad (14\text{-}3)$$

with

$$-RT \ln K = n(\mu_P^\circ - \mu_W^\circ) \qquad (14\text{-}4)$$

If n is large, this equation leads to an extremely steep isotherm, of the kind illustrated by portion *b* of Fig. 14-1. The transition from $\bar{\nu} = 0$ to $\bar{\nu} = n$ occurs over a very narrow range of unbound ligand concentration. The value of X_W at the midpoint ($\bar{\nu} = n/2$) is readily determined. At this midpoint $X_W = 1/K^{1/n}$ and $RT \ln X_W = \mu_P^\circ - \mu_W^\circ$.

When intrinsic cooperative binding occurs with hydrophobic ligands, the simultaneous participation of several ligand molecules in the binding process will most likely arise from a need for contacts between the hydrocarbon moieties of adjacent ligand molecules to achieve a stable product, and only a small proportion of the ligand molecules need to be in direct contact with the protein surface if n is large. In this situation the value of $\mu_P^\circ - \mu_W^\circ$ is more easily predictable than for binding of single ligands at discrete sites. For amphiphiles, for example, unless there are strong specific interactions involving the head groups, the free energy gain derived from removing the hydrophobic surface of the protein from water would be distributed among all ligand molecules if $\mu_P^\circ - \mu_W^\circ$ is defined by equation 14-4. The contribution per ligand would be small and $\mu_P^\circ - \mu_W^\circ$ would be expected to be quite close to the free energy change for self-association in the absence of protein, that is, close to $\mu_{\text{mic}}^\circ - \mu_W^\circ$.

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[1] The term *negative* cooperativity is sometimes used for broadening of the binding isotherm by repulsive interaction between sites. In that case the cooperativity discussed here would be called *positive* cooperativity.

Micellar Binding

A special case of intrinsic cooperativity occurs when a protein has a hydrophobic tail or belt, which can serve as a nucleus for formation of an amphiphile micelle. In that case equations 14-3 and 14-4 would apply with n approximately equal to the average aggregation number ($\bar{m}$) for formation of micelles in the absence of protein. Protein complexes would be expected to comprise a distribution of individual species with different values of m, but the effect of this on the experimental isotherm when $\bar{m}$ is reasonably large is insignificant, just as it was shown to be in Chapter 7 for consideration of the cmc and free energy of micellization of pure micelles. Since equation 14-3 depends only on the existence of PL_n (in this case with $n \simeq \bar{m}$) at equilibrium, the isotherm cannot distinguish between alternate mechanisms for formation of the micellar complex, that is, whether it is formed by aggregation of monomeric ligand on the protein surface or whether the protein inserts itself into a preformed micelle. In fact, it should be pointed out that binding isotherms cannot distinguish between micellar complexes and other forms of PL_n in which n happens to be close to $\bar{m}$. The distinction is conceptually convenient, but it cannot be claimed that any complex called "micellar" in later chapters has been experimentally characterized to demonstrate that the amphiphiles are actually arranged as in a protein-free micelle.

Cooperative Binding with Major Conformational Change

A cooperative binding isotherm can arise in quite a different way, when the binding sites do not exist on the native protein at all, but are created only by an unfolding of the native structure in which previously buried hydrophobic amino acid side chains are exposed (p. 144). Two successive equilibria are involved in this situation. The first is between the native protein (P) and its unfolded conformation (Q), and this equilibrium must lie far to the side of P since this is the stable form in the absence of the ligand: the standard free energy change ΔG°_{PQ} for conversion of P to Q must be positive, and it can be conceived to be as large as, say, 50 kcal/mol of protein. The second equilibrium is the equilibrium for complex formation between Q and the ligand L, which can involve discrete sites on Q for single ligands (with formation of QL, QL_2, and so on, if the equilibria could be observed without interference from the fact that the unliganded protein exists as P rather than Q), or complex formation can be intrinsically cooperative, with QL_n as the only possible product. Mathematical analysis of the effects of the combined equilibria (Foster and Aoki, 1958; Reynolds et al., 1967) shows, however, that the binding isotherm will usually be highly cooperative regardless of the mode of association with Q, because gross conformational changes of proteins are usually highly cooperative and unable to occur in stepwise fashion. If Q has discrete sites for single ligands, the binding of ligand to only a few of them would not be enough to compensate for the free energy change required to convert P to Q, and this conversion can therefore occur only at a ligand concentration sufficiently high to cause saturation or near saturation of all sites on Q. The binding process thus corresponds closely to the reaction $P + nL \rightleftharpoons QL_n$, and this would obey equation 14-3. The free energy change related to the equilibrium constant K would, however, include the free energy of unfolding as well as that for binding, that is,

$$-RT \ln K = \Delta G^\circ_{PQ} + n(\mu^\circ_Q - \mu^\circ_W) \tag{14-5}$$

where $\mu^\circ_Q - \mu^\circ_W$ is the gain in unitary potential for transferring ligand from aqueous solution to discrete binding sites or to an intrinsically cooperative binding region on the protein molecule in the unfolded state Q.

The isotherms for this type of association are of course indistinguishable from isotherms for intrinsic cooperative binding to the protein in its native state. Distinction between the two processes depends on recognition of drastic conformational change of the protein by spectroscopic and/or hydrodynamic methods.

Stepwise Association

Actual isotherms often consist of successive stepwise increments in $\bar{\nu}$, as illustrated by Fig. 14-1. If all such steps were noncooperative and if there were no evidence for conformational change at any stage, the situation would be analogous to that existing in the binding of protons to proteins (Tanford, 1961, chapter 8), and the most likely interpretation would be that each step represents binding to a set of n_i discrete binding sites, each

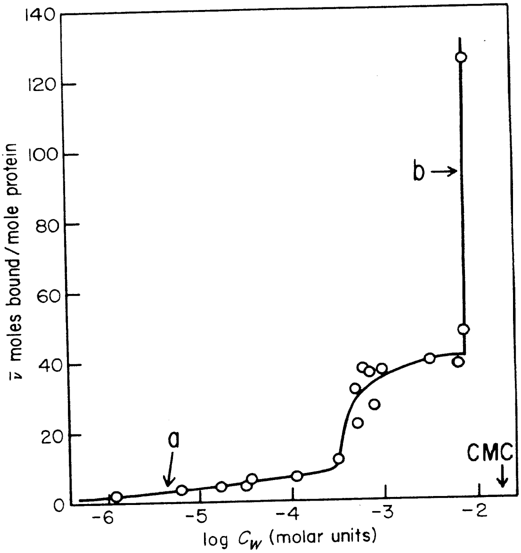

Fig. 14-1. Binding isotherm for the association of decyl sulfate with bovine serum albumin, at 2°C, pH 5.6, and ionic strength 0.03. The number of amphiphile ions required to saturate the protein in the final cooperative process has not been established. Data used for this figure were obtained by equilibrium dialysis and are given in different form by Reynolds et al. (1967). The concentration of unbound ligand (C_W) determined experimentally is the *total* concentration, but below the cmc this is not significantly different from the monomeric concentration, as shown by Fig. 7-1.

succeeding set having weaker affinity for the ligand. Apart from possible uncertainty in the evaluation of the precise values of n_1, n_2, and so on, because of overlap between the successive steps, the analysis of the data from each step would be no more complicated than for an isotherm consisting of a single sigmoid curve. When some of the steps are cooperative and/or accompanied by conformational change that can be detected by optical and hydrodynamic methods, the simplest and customary procedure is still to treat each successive step as phenomenologically distinct. In this situation, however, one cannot know whether the increase in the number of binding sites in a particular step is to be considered a true increment (previously filled higher affinity sites remaining unaffected) or whether it represents a process in which sites of higher affinity are abolished by the conformational change, all n sites now representing a new mode of binding. Equations allowing for both possibilities, as well as for the possibility that ΔG°_{PQ} may be small enough to allow for binding with conformational change to occur with only partial saturation of sites on Q, are given by Reynolds et al. (1967).

COMPETITION BETWEEN BINDING AND SELF-ASSOCIATION

Competition between several ligands for the same protein binding sites is a familiar phenomenon that can be recognized without difficulty by varying the kind and amount of possible competing substances (e.g., buffer ions) in the medium. It can be avoided in the study of a particular ligand by eliminating competing substances from the medium. Substances that form complexes with the ligand, in competition with protein-ligand binding, can be dealt with in the same way. In the study of the binding of hydrophobic ligands there is an entirely different competing process: self-association, which cannot be avoided. Self-association may take the form of phase separation (for hydrocarbons or alcohols) or of micelle formation, and its effect is to limit the attainable monomer concentration of ligand (X_W). If phase separation is the competing process, there is an abrupt cutoff when X_W reaches the saturation value. If micelle formation is the competing process, a rigorous treatment would require explicit consideration of the equilibrium between L in monomeric and micellar forms, and calculation of the slow increase in X_W which occurs when the total unbound ligand concentration is increased. As shown by Fig. 7-1, however, the increase in X_W with increasing total ligand concentration is sufficiently small to allow qualitative estimation of the effect of competition from micelle formation by treating it too as if it were a phase separation, with an abrupt cutoff in X_W when the cmc of the micelle-forming ligand is attained.

The general effect of competing self-association of ligand is thus to make it impossible to observe portions of the theoretical binding isotherm that lie at X_W above the cut-off value for the latter, as is illustrated by Fig. 14-2. In terms of free energy this means that only half or less of a noncooperative binding isotherm can be observed unless $-(\mu_P^\circ - \mu_W^\circ)$ *exceeds* the free energy gain in the competing process (Fig. 14-2*a*); if the binding is highly cooperative it cannot be observed at all unless the free energy gain on binding to the protein exceeds that of the competing process (Fig. 14-2*b*).

It is evident that this competitive feature of the binding process effectively limits the acquisition of analyzable data to situations where the free energy gain associated with binding to the protein exceeds the free energy gain in competing processes. If a binding isotherm is observable at all, the affinity of the ligand for the protein must be high in comparison with other processes previously considered. If binding cannot be observed, we can-

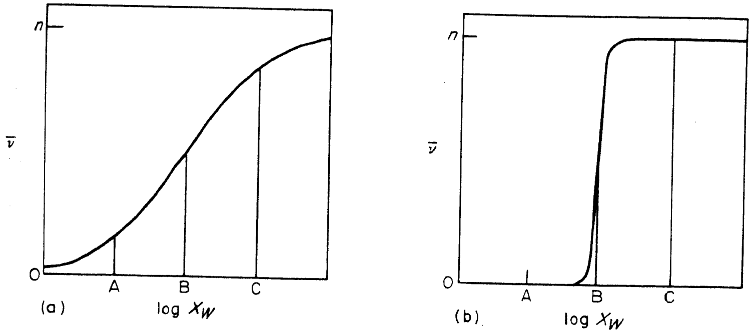

Fig. 14-2. Schematic diagrams of binding isotherms in relation to the maximum value of X_W attainable by virtue of self-association processes such as micelle formation. A represents the limiting value for X_W when $\mu^\circ_{\text{mic}} - \mu^\circ_W$ is about 1 kcal/mol more negative than $\mu^\circ_P - \mu^\circ_W$, B when the two are equal, and C when μ°_W is about 1 kcal/mol more positive than $\mu^\circ_P - \mu^\circ_W$. (*a*) Noncooperative; (*b*) highly cooperative.

not say that there are no binding sites on the protein, but only that $\mu^\circ_P - \mu^\circ_W$ or $\mu^\circ_Q - \mu^\circ_W$ for binding sites is not negative enough to compete effectively with self-association processes. For cooperative processes, less than 1 kcal/mol in $\mu^\circ_P - \mu^\circ_W$ and $\mu^\circ_Q - \mu^\circ_W$ can make the difference between ability to observe full saturation of the available sites and the absence of any observable binding at all.

It should be noted that $\mu^\circ_P - \mu^\circ_W$ and $\mu^\circ_Q - \mu^\circ_W$ *include* contributions to the free energy of association derived from the exposed hydrophobic binding site or region on the protein molecule (without which there would be no tendency for the protein to associate with the ligand at all), and that they will therefore generally be more negative than the free energies for self-association (only slightly so for intrinsically cooperative binding, cf. p. 149) if the ligand in the protein complex is as well shielded from contact with water, and if hydrocarbon chains are as mobile, as they would be in the competing self-associated state.

An additional competitive element for ligand binding can be introduced by self-association of the protein. This can stabilize the ligand-free state of the protein and can increase the value of $-(\mu^\circ_P - \mu^\circ_W)$ or $-(\mu^\circ_Q - \mu^\circ_W)$ required for ligand binding to be observable.

BINDING OF HYDROCARBONS TO β-LACTOGLOBULIN AND OTHER PROTEINS

One of the few examples of significant binding of hydrocarbons to simple proteins is provided by the work of Wishnia and co-workers (Wishnia and

Pinder, 1966; Robillard and Wishnia, 1972) on β-lactoglobulin. Both aliphatic and aromatic hydrocarbons were examined; only the data for the small alkanes, butane and pentane, are discussed here. Binding was determined from relative solubilities of these hydrocarbons in aqueous solutions with and without added protein. The protein was found capable of binding only two molecules of these hydrocarbons, and competitive binding studies showed that the same sites were used by both ligands. Equation 14-1 was obeyed for butane, suggesting that the two sites are identical, but the data for pentane required a lesser affinity for the second molecule than the first. The interpretation suggested by Wishnia and Pinder is that the protein has a single hydrophobic site, able to accommodate hydrocarbon with a molar volume of 200 to 230 cc. This provides a comfortable fit for two butane molecules, but a rather tight one for two pentane molecules. Nonadherence of the binding of the latter to equation 14-1 is thus ascribed to steric interference with binding of the second pentane molecule, the two halves of the hydrophobic patch constituting intrinsically identical sites. With this assumption, $\mu_P^\circ - \mu_W^\circ$ values can be obtained for pentane as well as butane by equation 14-1, provided one limits oneself in the case of pentane to results at low $\bar{\nu}$ where the fraction of protein molecules with two bound ligands is insignificant. The results obtained are shown in Table 14-1. The interpretation in terms of a single hydrophobic binding site is supported by the fact that β-lactoglobulin binds one molecule of dodecyl sulfate with very high affinity (Ray, 1968), roughly consistent with the value of $\mu_P^\circ - \mu_W^\circ$ expected on the basis of Table 14-1 for a molecule of *n*-decane.

In Table 14-1 the values of $\mu_P^\circ - \mu_W^\circ$ are compared with the values of $\mu_{HC}^\circ - \mu_W^\circ$ for the same hydrocarbons, taken from Table 4-1. It is seen that $\mu_P^\circ - \mu_W^\circ$ is somewhat more negative, as it has to be, since experimental data in excess of 50% saturation were obtained. In molecular terms, this presumably means that the process of binding not only removes the bound hydrocarbon from an aqueous environment, but also has a beneficial

Table 14-1. Free Energy of Association of Hydrocarbons with β-Lactoglobulin at 25°C[a]

	$\mu_P^\circ - \mu_W^\circ$ (kcal/mol)	$\mu_{HC}^\circ - \mu_W^\circ$[b] (kcal/mol)
Butane	−6.54	−5.97
Pentane	−7.60	−6.85

[a] From Robillard and Wishnia (1972).
[b] From equation 2-3.

effect on the apolar side chains of the protein. One model that would satisfy this requirement is a cleft in the protein surface which, in the absence of bound hydrocarbon, possesses a hydrocarbon–water interface. One would expect that the protein-bound hydrocarbon might be more rigid than the dissolved molecule and this alone would make $\mu_P^\circ - \mu_W^\circ$ more positive than $\mu_{HC}^\circ - \mu_W^\circ$: the fact that the difference between $\mu_P^\circ - \mu_W^\circ$ for butane and pentane is somewhat less than the difference in $\mu_{HC}^\circ - \mu_W^\circ$ is perhaps an indication of this. This and other minor factors mentioned on page 143 all lead to the conclusion that the difference of 0.3 to 0.5 kcal/mol between μ_P° and μ_{HC}° in Table 14-1 gives only a lower limit for the contribution of the amino acid side chains of the protein to $\mu_P^\circ - \mu_W^\circ$; the actual contribution is likely to be higher, say ~2 kcal/mol. Even this would be a small figure, comparable to the contribution from a single leucine residue completely surrounded by water (Table 13-1). It is more likely that several hydrophobic side chains are involved in creating the binding site, but that they are only partially exposed to the solvent. (It should be noted that ΔH°, ΔS°, and ΔC_p° for the association were also measured in these studies. The values of ΔC_p° are strikingly similar to the values for transfer of butane and pentane to liquid hydrocarbon, as given in Table 4-1.)

Among other proteins that have been investigated, ribonuclease and lysozyme (Wishnia, 1962) do not bind hydrocarbon at all at the hydrocarbon concentration to which one is limited. Serum albumin can bind several molecules of small hydrocarbons (Wetlaufer and Lovrien, 1964; Wishnia and Pinder, 1966), but the isotherms have the form illustrated by Fig. 14-2*a*, with the cutoff at point A, indicating that the binding reflects low levels of saturation of a fairly large number of sites of weak affinity for hydrocarbon. It is clear that hydrophobic surface patches of even such limited size as that possessed by β-lactoglobulin do not occur commonly on water-soluble proteins.

BINDING OF AMPHIPHILES TO SERUM ALBUMIN AND OTHER PROTEINS

There is a large body of experimental data on the binding of amphiphiles to proteins, which has been reviewed in detail by Steinhardt and Reynolds (1969). The majority of such studies have involved the binding of ionic amphiphiles to a single protein, serum albumin; this protein functions biologically as a carrier for fatty acid anions and other simple amphiphiles. Experimental studies of the association, as illustrated by Fig. 14-1, have generally yielded complex results, indicating several levels of affinity. The limited data available for other proteins indicate that binding with very high affinity may be nearly unique to serum albumin; several other common water-soluble proteins do not show it. The lowest affinity type of association, however, which proves to be a cooperative process in which protein can bind more than its own weight of amphiphile, appears to be a nonspecific process common to all proteins. We shall confine the discussion here to these extremes of the binding isotherm (i.e., regions *a* and *b* of Fig. 14-1).

High Affinity Sites of Native Serum Albumin

There are about 10 or 11 discrete binding sites with a high association constant for anionic amphiphiles, and considerable information concerning them is available, most of it from the work of Reynolds et al. (1967, 1968, 1970). Most of the sites are specific for ligands with anionic head groups, but there are four sites for binding cationic amphiphiles with moderate affinity (Nozaki et al., 1974), and the hydrophobic area of these sites may be shared with some of the sites for anionic ligands, as illustrated schematically in Fig. 14-4. Aliphatic alcohols bind to about half the sites, but with considerably less affinity than anionic molecules with hydrocarbon chains of the same length (Reynolds et al., 1968), and hydrocarbons alone bind only weakly, as previously mentioned. Direct evidence for active involvement of the anionic head group and selectivity for specific head groups is provided by the finding of Reynolds et al. (1968) that some of the binding sites, identifiable by perturbation of the tryptophan spectrum when binding takes place, do not bind alkyl carboxylates at all (or do so with greatly diminished affinity), whereas both alkyl sulfates and sulfonates are strongly bound.

It is well known that serum albumin is capable of combining with a large variety of anions, including such simple ions as Cl^-, SCN^-, and acetate. Quantitative binding isotherms were published as early as 1950 (Scatchard et al., 1950), and showed that a large number of sites were available on the protein, but that about 10 of these have higher affinity than the rest. No proof exists that these are the same sites as those to which anionic amphiphiles bind, but the numerical coincidence may not be fortuitous. It should be noted that the binding constants for these simple ions are several orders of magnitude smaller than those for binding of amphiphilic anions and that the separation of sites into affinity classes is more difficult. Later data of higher precision (Scatchard et al., 1957, 1959; Scatchard and Yap, 1964) indicate that one of the high affinity sites is a much stronger binding site than the rest, and that binding to this site is remarkably endothermic. There is a similar unique site for the binding of anionic amphiphiles (Goodman, 1958), and the ΔH for association of dodecyl sulfate with this site is -18 kcal/mol (Lovrien and Sturtevant, 1971).

In calculating $\mu_P^\circ - \mu_W^\circ$ for the binding of amphiphiles we have ignored the one exceptionally strong site because accurate data for this site (i.e., precise X_W values at $\bar{\nu}$ values <1) are extremely difficult to measure and have not been obtained. Average values of $\mu_P^\circ - \mu_W^\circ$ (page 148) have been calculated for the remaining sites, and some of them are shown in Fig. 14-3. They are of course much more negative than the values of $\mu_{\text{mic}}^\circ - \mu_W^\circ$ for the same substances, as is evident from a comparison (Fig. 14-1) of the free ligand concentration required to saturate the high energy sites and the critical micelle concentration. This greater free energy gain is ascribable to the contribution from the interaction between the amphiphile head group and the protein's anion-binding sites, a conclusion required by the experimental data regardless of whether the binding sites here have a one-to-one correspondence with the *strongest* general anion binding sites. It is not unreasonable to assign 3 to 4 kcal/mol to this interaction, which would mean that only about one-half of the value of $\mu_P^\circ - \mu_W^\circ$ may result from interaction with hydrophobic surface patches of the protein. This, and the inability to bind hydrocarbons such as butane and pentane to a significant degree, may indicate that the hydrophobic part of the binding site may not be a single contiguous area, but that it may

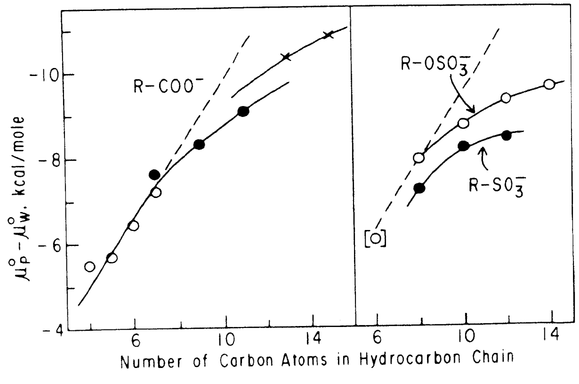

Fig. 14-3. Free energy of association between anionic amphiphiles and native bovine serum albumin (from Tanford, 1972). For $RCOO^-$ ○ data of Teresi and Luck (1952), 1°C, ionic strength 0.2; ● data of Reynolds et al. (1968), 2°C, ionic strength 0.03; × data of Spector et al. (1971), 37°C, ionic strength 0.15. For $ROSO_3^-$ and RSO_3^- all data are from Reynolds et al. (1967), 2°C, ionic strength 0.03, except that the point for hexyl sulfate is a result of poorer precision, taken from Steinhardt and Reynolds (1969). The dashed lines show the *slopes* of plots of $\mu_{HC}^\circ - \mu_W^\circ$ or $\mu_{ROH}^\circ - \mu_W^\circ$ versus n_C as given in Chapter 3.

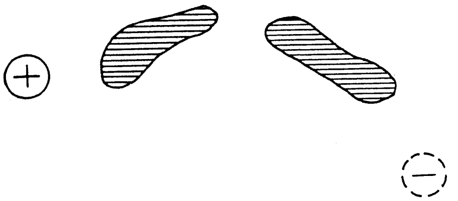

Fig. 14-4. Schematic diagram of a binding site of native serum albumin for amphiphiles. The hydrophobic patch (hatched area) is shown as broken into regions because neither butane nor pentane can be bound effectively. The two regions are placed at an angle to each other because at least one unsaturated fatty acid anion is bound with greater affinity than its saturated analog. The site marked with a plus sign represents one of the general anion binding sites of serum albumin; its juxtaposition to the hydrophobic patches is an essential element of the site to account for the fact that anionic amphiphiles are bound strongly whereas hydrocarbons are not. A small number of binding sites with moderate affinity for cationic amphiphiles exists, indicating that some of the binding sites may also have a negatively charged position near the hydrophobic areas as shown by the circle marked with a minus sign.

involve separated areas such as those illustrated schematically by Fig. 14-4. Binding to a site of this type would involve an "inch-worm" type of attachment of the ligand. Other evidence supporting a binding site such as is illustrated by Fig. 14-4 is provided by the fact that oleate (containing one centrally located double bond) binds more strongly to some of the binding sites than its fully saturated analog, stearate (Goodman, 1958; Spector et al., 1971), even though double bonds decrease hydrophobicity in general (Chapter 2), and $\mu^\circ_{HC} - \mu^\circ_W$ is less negative for aleate than for stearate (page 16). This may indicate that the central portion of the hydrocarbon chain does not contribute to the free energy of binding and that the hydrophobic binding areas are arranged so that a bend in the chain at the center, such as a double bond would introduce without loss of rotational flexibility, is helpful in the binding process.

Regardless of the exact nature of the binding process, the effect of chain length on $\mu^\circ_P - \mu^\circ_W$, as shown by Fig. 14-3, unequivocally demonstrates that the hydrophobic parts of the binding sites are circumscribed and limited in the length of hydrocarbon chain that can be accommodated. The dashed lines of Fig. 14-3 are drawn with a slope equal to the slopes of Fig. 3-1 and represent the expected effect of hydrocarbon chain length on $\mu^\circ_P - \mu^\circ_W$ arising from removal of the hydrocarbon chain from water. The experimental dependence of $\mu^\circ_P - \mu^\circ_W$ on n_C is seen to be of the right order of magnitude up to $n_C \simeq 8$, but a pronounced decrease is observed for $n_C > 8$. It evidently becomes increasingly difficult to find suitable locations for the hydrocarbon chain as its length increases, and little or no gain in free energy of binding occurs above $n_C = 12$.

It is of interest, in conclusion, to note the displacement by about one carbon atom of the positions of the curves for alkyl sulfates and sulfonates in Fig. 14-3. The underlying reason is presumably the same as that for the similar difference in the cmc values for these molecules, as discussed on page 68.

Massive Cooperative Binding Accompanied by Drastic Conformational Change

The cooperative formation of stoichiometric complexes between serum albumin and dodecyl sulfate, containing a very large number of bound ligands, was first recognized by Putnam and Neurath (1945). Formation of these complexes and of similar complexes by many other ionic detergents[2] with long hydrocarbon chains is accompanied by large changes in optical and hydrodynamic properties, indicative of a major conformational change (Reynolds et al., 1967; Steinhardt et al., 1971; Nozaki et al., 1974). In contrast to what was observed for binding of anionic amphiphiles to single-ligand sites on native serum albumin, there appears to be no limitation in the length of hydrocarbon chain (at least to $n_C = 14$) that can be accommodated in this type of complex with a gain in free energy. The parameter obtained from the experimental data (equation 14-4) is $\Delta G^\circ_{PQ}/n + \mu^\circ_Q - \mu^\circ_W$, and the values obtained from the data of Reynolds et al. (1967) at 2°C are -8.0 ± 0.5 kcal/mol for myristyl sulfate and -6.8 ± 0.5 kcal/mol for dodecyl sulfate. If $\Delta G^\circ_{PQ}/n$ is the same for the two ligands, the difference corresponds to an increment in $\mu^\circ_Q - \mu^\circ_W$ per carbon atom that is within experimental error the same as the increment of -700 cal/mol per carbon atom for micelle formation by the same substances.

It has been established (for dodecyl sulfate) that this type of complex can be formed with all proteins, and that the maximum amount of ligand that can be bound per g protein is nearly the same for many of them (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970a). For proteins without disulfide bonds, or with disulfide bonds reduced if originally present, there are actually two forms of the complex, as can be seen

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[2] The cooperative association between octyl and decyl sulfates and bovine serum albumin at 4°C appears to occur *without* drastic conformational change (Reynolds et al., 1967). For human serum albumin at higher temperatures the same result is obtained for octyl sulfate, but binding of decyl sulfate induces denaturation (Steinhardt et al., 1974; Krogh-Hansen and Riison, 1976). The mechanism whereby massive binding can occur without extensive unfolding of the native structure of the protein is not understood.

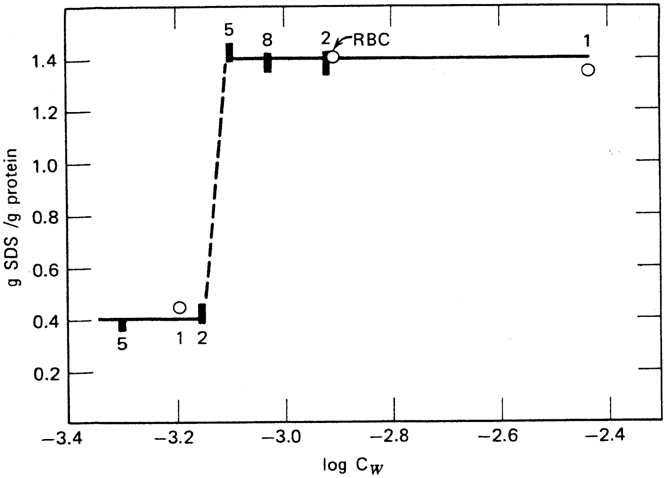

Fig. 14-5. Binding of dodecyl sulfate to proteins at 20°C, with disulfide bonds reduced where present in the native state. Similar results were obtained for a large variety of proteins, and the number adjacent to each point represents the number of different proteins used to obtain the result at each value of C_W. The point labeled "RBC" represents a mixture of proteins extracted from erythrocyte membranes. Measurements were made over a broad range of ionic strength with consequent variation in the cmc of the detergent. Open circles represent results where C_W is below the cmc. Filled rectangles represent results where the total concentration of uncombined dodecyl sulfate is above the cmc, so that micelles were present: C_W represents the concentration of monomeric ligand, equal to the cmc at the ionic strength employed. The half-filled rectangle represents experimental data with and without micelles at the same value of C_W (from Reynolds and Tanford, 1970a).

from Fig. 14-5, one containing 0.4 g dodecyl sulfate per g protein (one amphiphile per seven amino acid residues), and the other containing 1.4 g dodecyl sulfate per g protein (one amphiphile per two amino acid residues). The transition between them takes place at 6 to 7 × $10^{-4}M$ dodecyl sulfate at 25°C, pH 5.6 to 7.2, with little dependence on ionic strength. All proteins are dissociated to their constituent polypeptide chains, and each chain forms an individual asymmetric particle, approximately rod shaped, with length roughly proportional to molecular weight (Reynolds and Tanford, 1970b).[3] The particle may consist of short rigid rodlike segments, with intervening regions possessing some flexibility, as suggested on p. 144. (See also Shirahama et al., 1974; Rowe and Steinhardt, 1976.) Results obtained for proteins with intact disulfide bonds indicate that very similar complexes are formed, subject only to steric limitations imposed by the disulfide bonds. The amount of detergent bound is diminished (Pitt-Rivers and Impiombato, 1968) and the resulting complex is not as extended (Fish et al., 1970).

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[3] Complex formation with sodium dodecyl sulfate provides the basis for polypeptide chain analysis by electrophoresis on polyacrylamide gels (Weber and Osborn, 1975). Both molecular charge and sieving through the gel pores affect the mobility of polypeptides on the gel, and both would vary uniformly with molecular weight if the amount of bound dodecyl sulfate

Cationic amphiphiles bind cooperatively to most proteins to form complexes similar to those formed by dodecyl sulfate ions, but with diminished affinity (Nozaki et al., 1974). Nonionic amphiphiles with polyoxyethylene head groups, however, do not bind at all in the cooperative mode here described, and they can associate only with proteins that have appropriate binding sites or regions in the native state (Makino et al., 1973).

As noted earlier, it is not possible to distinguish between intrinsically cooperative ligand binding and binding of single ligands to discrete sites when the binding is to a *grossly altered form* of the protein, formation of which is induced by the association with ligand. There is good evidence that the binding of dodecyl sulfate ions and other ionic amphiphiles described here is intermediate between these extremes, involving patches of hydrophobic surface on the unfolded form of the protein which also contain ionic amino acid side chains with a charge opposite to that of the amphiphile being bound. That several amphiphiles bind simultaneously to each patch, with formation of a hydrophobic region containing many hydrocarbon chains in contact with each other, is demonstrated by the ready association of the protein–amphiphile complexes with water-insoluble dyes, which in the absence of protein can be solubilized by amphiphile micelles, but not by amphiphile in monomer form (Pitt-Rivers and Impiombato, 1968; Steinhardt et al., 1974). The extended shape of the protein–amphiphile complexes and probable involvement of ionic amino acid side chains suggest, however, that there must be many patches per molecule and that the number of amphiphiles bound at any one of them is much less than the total number (n) bound per protein molecule.

Participation of charged amino acid side chains in the binding process is dictated by thermodynamic considerations. Since binding competes successfully with micelle formation, the net free energy change per am-

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per g protein and the rodlike shape were the same for *all* polypeptides. In that case polypeptides would separate in gel electrophoresis strictly on the basis of molecular weight, but in fact exceptions to the general mode of association with sodium dodecyl sulfate are not infrequent, and apparent molecular weights based on electrophoretic mobility are properly regarded as representing only preliminary estimates.

phiphile, which by equation 14-4 has an average value of $\Delta G^\circ_{\mathrm{PG}}/n + \mu^\circ_{\mathrm{Q}} - \mu^\circ_W$, must be more negative than $\mu^\circ_{\mathrm{mic}} - \mu^\circ_W$. With $\Delta G^\circ_{\mathrm{PQ}}$ necessarily positive, this means that $\mu^\circ_{\mathrm{Q}} - \mu^\circ_W$ must be *substantially* more negative than $\mu^\circ_{\mathrm{mic}} - \mu^\circ_W$. Micelle formation by ionic amphiphiles is *impeded* by repulsive interaction between charged head groups, and the presence of a charge of opposite sign at the binding region of the protein can readily lead to the required difference between μ°_{Q} and μ°_{mic}, especially if the number of amphiphiles associated with each binding patch is relatively small.

The inability to observe cooperative binding with denaturation when nonionic amphiphiles are used supports this explanation. Nonionic amphiphile micelles are not subject to electrostatic repulsion between head groups. As seen from Fig. 7-3, they have cmc values an order of magnitude lower than ionic amphiphiles with hydrocarbon chains of the same length, and correspondingly more negative values of $\mu^\circ_{\mathrm{mic}} - \mu^\circ_W$. There is no easily conceived mechanism whereby $\mu^\circ_{\mathrm{Q}} - \mu^\circ_W$ can now become substantially more negative than $\mu^\circ_{\mathrm{mic}} - \mu^\circ_W$. The proposed mechanism also accounts for the observation that cooperative binding with denaturation requires a higher free amphiphile concentration for cationic than for anionic amphiphiles. The amino acid side chains creating favorable electrostatic interaction for cationic amphiphiles are glutamic and aspartic acid side chains, having two and one side chain CH_2 groups, respectively. The corresponding side chains for anionic amphiphiles are lysine and arginine, which have four and three side chain CH_2 groups, respectively, and can therefore make a significant hydrophobic contribution to $\mu^\circ_{\mathrm{Q}} - \mu^\circ_W$ as well as providing favorable electrostatic interaction.

CONCLUDING REMARKS

The binding studies cited here (and other work not mentioned) do not provide any examples of micellar or other intrinsically cooperative binding to the *native* state of common water-soluble proteins. Such binding, however, will be seen to be a characteristic property of proteins that exist in their native state in association with phospholipids, either as soluble lipoprotein complexes or as membrane proteins. Association with lipids thus appears to result from the presence of a hydrophobic region on the preferred folded structure of a protein that can associate cooperatively and often relatively nonspecifically with a variety of amphiphiles. As was first suggested by Helenius and Simons (1972), one can thus use cooperative association with nonionic amphiphiles as a criterion to identify a protein as lipophilic, since nonionic amphiphiles are not capable of cooperative association with common water-soluble proteins. Water-soluble proteins can combine cooperatively only with ionic amphiphiles capable of inducing unfolding of the native structure, and the suggestion made some years ago by Reynolds and Tanford (1970a) that this latter type of reaction might be similar to protein–lipid interaction of lipophilic proteins is clearly invalid.

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FIFTEEN

SERUM LIPOPROTEINS[1]

Soluble lipoproteins are not only of interest for their own sake, but they are also an essential topic for a discussion of biological membranes. An understanding of the interaction of lipids with proteins in the formation of membranes would not be complete unless we also understand how a similar type of interaction can lead to the formation of soluble particles with no tendency for incorporation into membranes. This chapter will summarize the present state of knowledge about the best studied proteins of this kind, the serum lipoproteins. Lipoproteins from egg yolk have also received a fair amount of attention (e.g., Cook and Martin, 1969; Ohlendorf et al., 1978).

The serum lipoproteins have been difficult to study because they are variable in composition. Variability with respect to the lipid component is an essential property because the principal physiological function of the lipoproteins is to transport lipids and to deposit them where they are needed, but there is also variability with respect to polypeptide chain composition, and some polypeptides are known to be transferred from one lipoprotein to another in the course of vascular circulation. There are significant differences between serum lipoproteins from different animal species, and the discussion here is limited to those from human blood.

Initial fractionation is usually achieved by flotation in salt solutions of different density, and four major fractions are recognized. In order of increasing density and protein content they are chylomicra, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The chylomicra are especially interesting. Their protein content is less than 2% and about 90% of the lipid is in the form of triglycerides (i.e., triacyl derivatives of glycerol). Since triglycerides themselves have extremely low solubility in aqueous solutions, the mechanism whereby they can be solubilized by so small an amount of protein is one of the most intriguing problems in the field of protein–lipid interactions. The very low protein content has, however, discouraged detailed investigation of the protein moiety from the chylomicron fraction, and considerably more progress has been made for the other fractions, where the protein/lipid ratio is higher. For this reason only the three denser fractions will be discussed here.

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[1] This chapter was written in collaboration with Dr. J. A. Reynolds.

Minor serum lipoproteins, not falling into the classes enumerated above, have been identified, often associated with particular clinical disorders. There are many excellent reviews, which describe the total spectrum of these proteins more fully than is possible here, and also review their functional and metabolic properties (e.g., Jackson et al., 1976; Lewis, 1977).

POLYPEPTIDE CHAINS

Because polypeptide chains can be exchanged between lipoproteins in different classes, a discussion of the molecular characteristics has to begin with an enumeration of the constituent polypeptides rather than with the native molecules. A list of the most abundant polypeptides is given in Table 15-1: one of them (D) is probably not a constituent of the major lipoproteins discussed in this chapter. Amino acid sequences have been determined for five of the listed polypeptides, and accurate molecular weights have been measured for two others. An interesting feature of the sequences is that none of them contains long continuous segments of hydrophobic amino acids, such as have been found in the sequences of some membrane proteins (Chapter 17). The very high molecular weight for the B-peptide is also noteworthy; this is one of the largest polypeptide chains known.

NATIVE LIPOPROTEINS

Table 15-2 lists analytical data for typical subfractions of the serum lipoproteins. The subfractions are still heterogeneous, but they have a narrower distribution with respect to molecular size and composition than whole fractions would have. All of the serum lipoproteins contain phospholipid (Skipsky et al., 1967), and the predominant components are phosphatidylcholine and sphingomyelin, both of which have the same

Table 15-1. **Major Polypeptide Chains of Human Lipoproteins**

Polypeptide[a]	Amino Acid Sequence Determined	Molecular Weight from Sequence	Molecular Weight by Ultracentrifuge[b]
A-I	Yes[c]	28,330	28,300
A-II	Yes[d]	8,710	9,100
B	No	—	255,000
C-I	Yes[e]	6,620	7,000
C-II	Yes[f]	8,840	10,000
C-III	Yes[g]	8,760	10,000
D[h]	No	—	(18,000)
E	No	—	33,000

[a] Nomenclature of Alaupovic et al. (1972).

[b] Measurements were made under conditions where complete dissociation to the monomer state is virtually certain. Data for the A-peptides are from Makino et al. (1974), for the B-peptide from Smith et al. (1972), for the C-peptides from Brown et al. (1970), and for the E-peptide from Shelburne and Quarfordt (1974). The figure for the B-peptide includes 7% bound carbohydrate. The figure for the D-peptide is based on amino acid analysis and does not include 18% bound carbohydrate (McConathy and Alaupovic, 1976).

[c] There are some unresolved differences between sequences obtained by Baker et al. (1974) and Brewer et al. (1978). The molecular weight given in column 3 is based on the sequence of Baker et al.

[d] Brewer et al. (1972). In humans this polypeptide is always present as a disulfide-bonded dimer.

[e] Shulman et al. (1972); Jackson et al. (1974).

[f] Jackson et al. (1977).

[g] Brewer et al. (1974). This polypeptide contains a carbohydrate side chain and exists in three forms containing, respectively, 0, 1, and 2 terminal sialic acid residues. The carbohydrate is not included in the molecular weight calculated from the sequence.

[h] Probably not a constituent of the principal native lipoproteins listed in Table 15-2.

zwitterionic head group (p. 107). The fatty acid chains are about 50% unsaturated, as in phospholipids derived from membranes.

It is seen from the data of Table 15-2 that C-peptides are present in all native proteins except LDL_2, but that A- and B-peptides never occur in the same molecule, the former being present only in HDL and the latter in LDL and VLDL. Recent data strongly suggest that the A- and B-peptides exist as two (and *only* two) core proteins, which are the nuclei for generation of all the major lipoprotein species other than chylomicra.

Table 15-2. Analytical Data for Native Lipoproteins[a]

	Molecular Weight	Weight %		Constituent Polypeptides[b]	Lipids (weight % of total lipid)[c]			
		Protein	Lipid		PL	FC	CE	TG
$VLDL_1$	32×10^6	6.4	93.6	B, C, E	11	6	8	75
$VLDL_4$	15×10^6	9.4	90.6	B, C, E	17	8	15	60
$VLDL_7$	8×10^6	13.2	86.8	B, C, E	23	10	26	41
LDL_1	4×10^6	18.0	82.0	B, C, E	29	7	28	36
LDL_2	2.6×10^6	21.0	79.0	B	29	10	53	8
HDL_2	360,000	40	60	A-I, A-II, C	53	10	29	8
HDL_3	176,000	55	45	A-I, A-II, C	54	8	28	10

[a] Data for VLDL are for representative subfractions from Patsch et al. (1978), for LDL they are from Lee (1976), and for HDL from Scanu (1971). Molecular weights are crude estimates from various sources.

[b] All three C-peptides are present when the presence of C is indicated.

[c] PL = phospholipid, FC = free cholesterol, CE = cholesteryl esters, and TG = triglycerides.

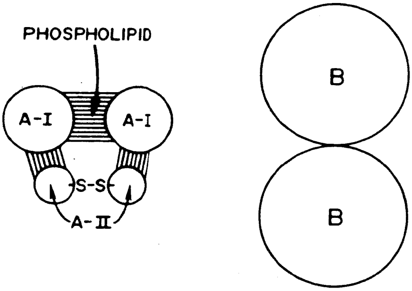

Fig. 15-1. Core proteins for HDL (mol. wt. 74,000, left) and for LDL and VLDL (mol. wt. 510,000, right). The A-II polypeptides in the HDL core protein are joined (in humans) by a disulfide bond. The shaded areas in the diagram for the HDL core protein are intended to symbolize an apparent requirement for the presence of diacyl phospholipids in the formation of this specific complex (see text).

HDL Core Protein

Friedberg and Reynolds (1976) have made a careful study of the stoichiometry of association between A-I and A-II in native HDL, utilizing a method that avoids differential loss of the two peptides in fractionation procedures. Employing 10 different normal subjects, and examining both HDL_2 and HDL_3, they observed a constant 1 : 1 ratio between A-I and A-II polypeptides, suggesting the existence of a core protein as shown in Fig. 15-1. Molecular weights reported for native HDL_3 range from 148,000 to 186,000, about half of which is protein, indicating that each HDL_3 molecule contains one copy of the core protein. The molecular weight of HDL_2 is probably close to 360,000, with a protein content of 40%, indicating the presence of two copies of the core protein. Both forms contain C-peptides, but at a level of less than 10% by weight. The average content of C-peptides in HDL_3 corresponds to less than one polypeptide chain per molecule, so that some HDL_3 molecules undoubtedly contain only the core protein and lipid.

LDL and VLDL Core Protein

It has been shown by Schumaker (1973) and Fisher et al. (1975) that variations in the molecular weight of LDL_2 between 2×10^6 and 4×10^6 probably arise from variations in the lipid content, the protein content remaining constant at 510,000 g/mol, that is, two B-polypeptides per molecule. The same is probably true for VLDL. For example, Kane et al. (1975) have used the insolubility of the B-polypeptide in tetramethyl urea to determine the amount of B-peptide in VLDL samples from 43 normal human subjects, with a mean particle diameter of 364 Å, which corresponds to a mean molecular weight of 14.1×10^6. B-peptide was found to represent 36.9% of the total protein. With a normal total protein content of 9% (Scanu et al., 1975), this corresponds to 470,000 g of B-peptide per particle, not including the 7% carbohydrate normally part of the B-peptide. If this is included, the total amount of B becomes 505,000. (The measured molecular weight of 255,000 for the B chain includes the carbohydrate moiety.) In subfractions of VLDL (Kane et al., 1975; Patsch et al., 1978) the content of B-peptide varies inversely with total molecular weight, such that the amount of B per particle remains constant to within the accuracy of analytical methods. The most compelling evidence for an invariant amount of B-peptide in all VLDL and LDL molecules comes from studies of lipoprotein catabolism and lipid metabolism (Lewis, 1977), which show that there is a precursor-product relationship between VLDL and LDL_2, with a progressive decrease in C-peptides and lipids (primarily triglycerides). The smaller VLDL molecules have been called "remnant molecules" and LDL_2 is the ultimate remnant, containing no C-peptides and the least amount of lipid.

C-Peptides

It is not yet known whether the C-peptides form core protein complexes of fixed stoichiometry. Since they are readily transferred between VLDL and HDL, they are certainly not tightly bound to the core proteins formed by A or B polypeptides, but it is not known whether the peptides are associated with each other or with lipid during the transfer process. One of the C-peptides (C-II) is not simply a lipid transport protein, but has a separate physiological function in being a potent activator of the enzyme lipoprotein lipase. (The work of Kostner and Alaupovic (1972) and Alaupovic et al. (1972) purports to show that C-peptides form inherently independent lipoproteins, and that native VLDL and HDL molecules are created by weak complex formation between the C-containing lipoproteins and lipoproteins containing only A- or B-peptides. This is not an unreasonable possibility, but their experimental evidence is not compelling.)

Two approaches can be employed to find out how the core proteins of Fig. 15-1 organize themselves and how they combine with C-peptides and lipids into the ultimate lipoprotein molecules: one can use structural information derived from investigation of the intact native proteins; or one can start with the purified polypeptide chains and determine by thermodynamic and structural methods the mode of their association with each other, with hydrophobic ligands in general, and with phospholipids and neutral lipids in particular. Information obtained by both methods is summarized in the remainder of this chapter.

STRUCTURAL INFORMATION

It was shown by Oncley and co-workers more than 30 years ago that LDL, despite its high molecular weight and high lipid content, has a compact, nearly spherical shape, much like that of common compactly folded globular proteins that do not contain lipid (Oncley et al., 1947). A similar result has since been obtained for VLDL and HDL as well, both by electron microscopy and by hydrodynamic studies (e.g., Nichols, 1969).

In another paper, Oncley et al. (1950) showed that the pH-dependence of the solubility of LDL resembles that of ordinary water-soluble proteins and could be ascribed to the titration of the acidic and basic groups of the protein moiety. Titration of most of these groups could be observed without indication of denaturation or other structural change, implying that most of the groups had to be at the surface of the native molecule, in contact with solvent. A similar inference can be made for HDL (at least for the amino groups) on the basis of their availability for succinylation (Scanu et al., 1968). Virtually all phosphate ester groups of phospholipid molecules in HDL and LDL are likewise accessible to solvent on the basis of shifts in the ^{31}P nmr spectrum induced by paramagnetic ions (Assmann et al., 1974; Yeagle et al., 1978). In HDL all phospholipid head groups have been shown to be available for cleavage by the enzyme phospholipase A_2 (Pattnaik et al., 1976). These results come as no surprise. The phospholipid head groups are ionic, and the titratable groups of proteins are ionic at neutral pH, and together they represent the most strongly hydrophilic parts of the molecular complex, which could be removed from contact with solvent only at great cost in terms of free energy (Chapter 13).

Dimensional considerations suggest that phospholipid head groups and protein polar groups completely fill the surface layer of a lipoprotein molecule, leaving no room for any part of the neutral lipid constituents (Shen et al., 1977). The neutral lipids thus occupy the internal space of the lipoprotein molecule, and this is not thermodynamically unreasonable. The ester groups of cholesteryl esters and triglycerides are only weakly hydrophilic in comparison with the charged groups present on ionic amino acid side chains of proteins and on phospholipid head groups. Moreover, the hydrophobic moieties on these molecules are extremely large, so that the free energy gained by their removal from contact with water far exceeds the free energy lost by removing the small hydrophilic groups along with them. A temperature-induced order–disorder transition has been observed in native LDL by several physical techniques (Deckelbaum et al., 1977a; Sears et al., 1976; Atkinson et al., 1977). It involves cholesteryl esters and has been interpreted as reflecting the presence of cholesteryl esters in LDL in a separate domain, with a molecular arrangement similar to that in pure cholesteryl esters. The presence of triglycerides lowers the temperature of the transition, indicating that triglycerides enter the same domain, being soluble only in the disordered phase. In VLDL, where the triglyceride content becomes very high, a transition can no longer be observed (Deckelbaum et al., 1977b).

The ^{31}P nmr studies of Yeagle et al. (1977, 1978) also provide information about vicinal interactions of the phospholipid head groups. In LDL about 20% of the groups are immobilized by interaction with the protein, but the specific interaction with cholesterol, as normally seen in cholesterol-containing phospholipid bilayers (p. 122), is absent, indicating that the OH group of unesterified cholesterol (like the ester groups of cholesteryl esters) may be located away from contact with solvent. In HDL there appear to be no immobilizing interactions between phospholipid head groups and protein.

Highly detailed structural models for HDL and LDL have been proposed (Assmann and Brewer, 1974; Mateu et al., 1972; Tardieu et al., 1976; Müller et al., 1978), but they are based more on imagination than on fact. The models for LDL rely in part on X-ray or neutron scattering data for lipoprotein solutions, but the scattering technique is powerful only when applied to systems with long-range order, such as the ordered phospholipid bilayers discussed in Chapter 11. Unrealistic simplifying assumptions are required when the technique is applied to solutions: for example, that a lipoprotein complex can be divided into two homogeneous and spherically symmetrical regions, one with low electron density ("hydrocarbon") and another with high electron density ("polar groups"). All the proposed models contain features that are inconsistent with other data cited in this chapter.

THERMODYNAMICS OF ASSEMBLY

The study of the assembly of the lipoproteins, starting with purified polypeptide chains, is easier for HDL than for LDL and VLDL because the A- and C-polypeptides are soluble in aqueous solutions in the absence of lipids. Furthermore, it has been shown that a lipoprotein particle closely resembling native HDL can be reconstituted by sonication of a mixture of HDL polypeptides and constituent lipids in appropriate relative amounts (Hirz and Scanu, 1970; Lux et al., 1972). The free B-polypeptides, on the other hand, are not water-soluble and step-by-step reconstitution with thermodynamic analysis of each step becomes more difficult. For this reason analysis of the assembly process has progressed further with HDL than with LDL and VLDL, and the discussion here is confined to HDL.

Self-Association of Apoproteins

A-I and A-II polypeptides are independently soluble in aqueous solutions, as fairly compact folded molecules called apoA-I and apoA-II. Circular dichroism studies indicate that the polypeptide chain conformation in both apoproteins is different from the conformations adopted by A-I and A-II when incorporated into native HDL (Lux et al., 1972). Both conformations have only marginal stability and are disrupted by denaturing agents or heating under conditions where the native lipoproteins are unaffected (Gwynne et al., 1975; Reynolds, 1976; Tall et al., 1976). The conformational adaptability suggested by these results may be important in relation to the ability of these proteins to combine with variable amounts of lipids.

Both apoA-I and apoA-II self-associate in aqueous solution (for review see Osborne and Brewer, 1977), but the association of apoA-I alone does not lead to a stable dimeric state, the state in which A-I occurs in the HDL core protein (Fig. 15-1), nor do experiments with mixtures of apoA-I and apoA-II indicate preference for formation of a complex with the stoichiometry of the core protein (Friedberg and Reynolds, unpublished results). When simple hydrophobic ligands are bound to apoA-I, there are spectral changes suggesting that the polypeptide chain approaches the conformation it has in the native lipoprotein, but ligand binding stabilizes the *monomer* state of the protein. However, when diacyl phospholipids are bound to A-I, a homogeneous product with a molecular weight corresponding to an A-I dimer is formed spontaneously (Reynolds et al., 1977), which suggests that the formation of the HDL core protein as a whole is intimately linked to the presence of diacyl phospholipids, as indicated schematically in Fig. 15-1. The self-association of apoA-I and apoA-II in aqueous solution in the absence of any hydrophobic ligand may be ascribable to the existence of hydrophobic areas on the apoproteins, intended for binding of hydrophobic ligands, which in the absence of such ligands, tend to associate with each other. A similar tendency for self-association has been observed for membrane proteins that can be dissolved in purely aqueous solutions to a measurable extent. An example is provided by the work of Calabro et al. (1976) with cytochrome b_5.

As noted earlier, apoB cannot be studied in the absence of ligands because of its insolubility in aqueous solutions, but, in contrast to A-I and

A-II, it has a strong tendency to exist as a dimer in detergents, even in a denaturing detergent such as Na dodecyl sulfate (Steele and Reynolds, 1979), indicating that formation of the B core protein (Fig. 15-1) is not dependent on the presence of diacyl phospholipids and may be an inherent property of the protein alone.

Binding of Simple Hydrophobic Ligands to A-I and A-II

There is good evidence that the binding of lipids to lipoproteins involves sites or regions of the protein specifically designed to accommodate the hydrophobic moieties of the lipid, and that interaction between the protein and the polar head groups of the lipids makes little contribution to the free energy of binding, at least in the case of HDL. (For example, see the nmr data cited above.) Under these circumstances it becomes useful to explore the nature of the binding sites by studying the association of the protein with simple ligands containing hydrocarbon chains, but lacking the complex head groups of the endogenous phospholipids.

Experimental binding data show that apoA-I and apoA-II display *both* noncooperative and cooperative binding of simple ligands (Reynolds and Simon, 1974; Makino et al., 1974; Stone and Reynolds, 1975; Haberland and Reynolds, 1975). Each protein has three or four discrete sites for binding single molecules of hydrophobic ligands. In contrast to serum albumin (Chapter 14), there is no head group specificity. Cationic and anionic amphiphiles bind equally well, and even pure hydrocarbons can be accommodated, the free energy of binding being in each case consistent with what is expected on the basis that hydrophobic interactions alone are the determining factor. Binding to the discrete sites induces a small conformational change in the protein, and cooperative binding of a large number of ligands occurs only after this change has occurred; synergistic effects have been noted, suggesting that occupancy of the discrete sites by any one ligand makes the cooperative sites available to all other ligands (Stone and Reynolds, 1975). Cooperative binding, like binding to the discrete sites, does not seem to involve head group specificity.

Perhaps the most important result of these studies has been to show that, for ligands containing normal hydrocarbon chains as the hydrophobic moiety, fewer molecules of ligand can be bound in the cooperative mode (to apoA-I) as the size of the hydrophobic moiety increases, suggesting that the binding site is designed to accommodate a fixed volume of hydrocarbon, about 24,000 cm^3 per mol of polypeptide chain. This result is quite different from what has been observed with membrane proteins. While there are far too few studies of membrane proteins to permit a conclusive generalization, the few results obtained (Chapter 17) suggest that intrinsic membrane proteins are designed to comicellize with micellar aggregates of amphiphiles in such a way as to cause minimal perturbation of the micellar aggregate, and the amount of amphiphile bound is therefore determined by the size that the amphiphile micelle would have in the absence of protein. The lipoproteins on the other hand (to the extent that one can judge from the data for A-I) package ligands in an amount and manner of organization dictated by the structure of the protein.

A graphic visualization of the difference between binding to apoA-I and binding to membrane proteins is shown by the gel exclusion chromatography experiments in Fig. 15-2. The ligand for apoA-I is palmitoyl lysophosphatidylcholine, which, under the conditions of the experiment, forms large aggregated micelles emerging close to the void volume of the column, whereas the apoprotein alone runs as a small particle with a Stokes radius (R_s) of 28 Å. When excess lysolipid is added to the protein, it is seen that a protein–lipid complex is formed which is larger than the lipid-free apoprotein (R_s = 35 Å), but much smaller than the pure micelle. Excess ligand appears in the void volume as before. Similar experiments done for at least one membrane protein (cytochrome b_5) show that the protein–ligand complex is always of the same size as or slightly larger than protein-free micelles (Robinson and Tanford, 1975).

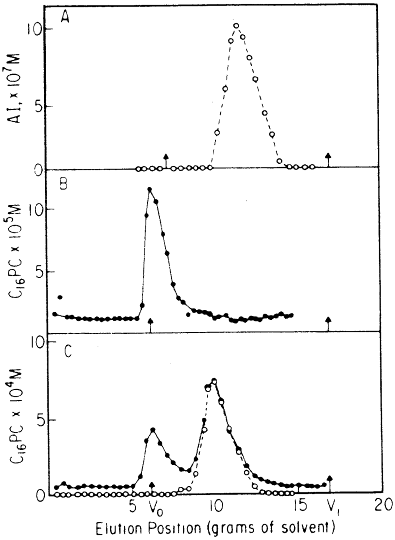

Fig. 15-2. Gel filtration chromatography on Sepharose 6B of apoA-I alone (*A*), palmitoyl lysophosphatidylcholine alone (*B*), and a mixture of the two (*C*). Open and filled circles represent analysis for protein and phospholipid, respectively, and arrows indicate void and total volume markers. From Haberland and Reynolds (1975).

Binding of Endogenous Lipids to ApoA-I

One quantitative binding study has been carried out using a diacyl phospholipid, di-decanoyl phosphatidylcholine (Reynolds et al., 1977). Apart from having shorter alkyl chains, this lipid is representative of the major class of endogenous phospholipids in HDL. The maximal amount bound was found to be consistent with the fixed volume binding region found for simpler amphiphiles, confirming the role of the protein in dictating the mode of association with lipid that was deduced from the data for simple amphiphiles. The same study also confirmed that binding to the discrete sites on A-I for single ligand molecules is necessary to trigger the conformational change that is a prerequisite for cooperative binding. The data showed, however, that the discrete sites appear to be specific for amphiphiles with *single* hydrocarbon chains and that diacyl phospholipids do not gain enough from the free energy of association to make binding to the protein favorable relative to self-association. A small number of single-chain amphiphiles must therefore be bound before association with diacyl phospholipids can occur; under physiological conditions they would presumably be fatty acids or lysophospholipids. Earlier qualitative studies (Assmann and Brewer, 1974; Verdery and Nichols, 1974) lead to similar conclusions. The functional significance, if any, of the need for monoacyl ligands is not known.

As noted earlier, the cooperative binding of diacyl phospholipids is accompanied by spontaneous association of A-I to the dimeric state. Since both diacyl and monoacyl molecules appear to utilize the same binding region, and the volume of bound hydrocarbon is about the same for both, the factor responsible for dimerization when diacyl phospholipids are bound may be the much greater separation between head groups for the same volume of hydrocarbon (Table 6-3), that is, the same factor that causes diacyl phospholipids alone to form extended bilayers instead of small soluble micelles.

It is known that neutral lipids (mostly cholesteryl esters in the case of HDL) do not combine with HDL until after diacyl phospholipids are bound (Sodhi and Gould, 1967; Scanu, 1972). The conformational adaptability of A-I and A-II is likely to be an important factor here, permitting association with *variable* amounts of neutral lipid, in contrast to the fixed amount of phospholipid that has to be associated with at least A-I. Detailed thermodynamic studies are not available.

CONCLUDING REMARKS

One of the objectives of this chapter has been to explore the difference between the hydrophobic binding sites of lipoproteins and of membrane proteins, the latter being designed for insertion into extended phospholipid bilayers without disruption of the bilayer organization, whereas lipoproteins are always found in nature as soluble particles. They come into frequent contact with cell membranes and transfer lipids to them, but the polypeptide chains always remain in solution. In the case of A-I the characteristics of the binding site (or sites) appear to be established by the data cited above: the protein is designed to accept a fixed volume of hydrocarbon in such a way that polar groups attached to the hydrocarbon face outward at the surface of the protein–ligand complex. It is likely that other soluble lipoprotein polypeptides share this structural feature, and direct evidence exists in the case of C-III. A study of the combination of C-III with dimyristoyl phosphatidylcholine (Novosad et al., 1976) showed that binding leads to disruption of the phospholipid bilayer and repackaging of the lipid to form protein–lipid complexes of relatively small particle size, similar to those shown to be formed in Fig. 15-2 in the interaction between A-I and lysophosphatidylcholine.

Other aspects of the assembly of HDL lipoproteins are still largely unexplored. The reason for the requirement that A-I must combine with a few molecules of ligands with a single alkyl chain before diacyl phospholipids can be bound is obscure, and it is not known whether this applies to A-II and the C-peptides as well. There are many reports in the literature that the A- and C-peptides (including A-I) combine directly with diacyl phospholipids, but the experiments usually involve cosonication of lipid and protein at high concentration, in the course of which some hydrolysis to lysophospholipid and fatty acid undoubtedly occurs. Whether or not these hydrolysis products are essential for the association with diacyl phospholipids can be determined only if thermodynamic binding studies are carried out as a function of progressively increasing lipid concentration, as was done in the study with A-I cited earlier.

A suggestion that all A- and C-polypeptides have a common mechanism for the binding of lipids comes from an analysis of their amino acid sequences, which shows that they all contain sequence segments that are capable of forming α-helical structures in which one face is hydrophobic while the opposite face is charged. Moreover, the charged faces so formed would contain positive and negative charges in about equal number in separate positive and negative strips (Segrest et al., 1974). Similar sequence segments occur in other proteins as well, but at a much lower frequency than in the lipoprotein polypeptides. The hypothetical helical structures that might be formed by these sequence segments have been called ''amphipathic helices,'' and it has been pointed out that they would be ideally designed for interaction with the endogenous lipoprotein phospholipids, most of which have zwitterionic head groups. However, they would appear to be especially well adapted for association with phospholipid in bilayer form (Segrest, 1977), whereas in fact A- and C-peptides do not enter membranous bilayers, at least under physiological conditions. Other evidence that raises questions about the significance of the postulated amphipathic helices comes from experimental data cited earlier in this chapter, that is, the lack of an effect of the head group on the binding of hydrophobic ligands to A-I and the fact that nmr measurements of native HDL show no perturbation of phospholipid head groups by the presence of the protein.

None of the available data at present provides any information about the manner of association of neutral lipids or C-peptides with HDL, and there is no knowledge at all of the assembly of LDL and VLDL from the B_2 core protein. The latter is an especially intriguing problem because the two B-polypeptides have the remarkable property of being able to pull together into a single lipoprotein particle as much as 50 times their own weight of lipid and small polypeptide (Table 15-2).

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SIXTEEN

BIOLOGICAL MEMBRANES

Biological membranes are thin layers of protein and lipid that permit the compartmentalization of living matter. The simplest example is the cytoplasmic membrane (also called "plasma membrane") that separates the contents of a single cell from its environment. An example is shown in Fig. 16-1. Simple membranes of this type surround all living cells. They serve as barriers to prevent the mixing of the contents of the cell with its surroundings, but at the same time must permit the selective passage of metabolites in and out of the cell.

The cells of higher organisms (eukaryotic cells) are much larger than the cells of bacteria and similar organisms (prokaryotic cells). They usually contain intracellular membranes that define compartments within the cell. The cell nucleus and mitochondria are examples, each being surrounded by its own membrane. Eukaryotic cells also contain endoplasmic reticulum, which is a network of membrane-bounded channels that traverse the entire cell. The mature human erythrocyte (Fig. 16-1) is thus an atypically simple type of eukaryotic cell, since it contains no nucleus or other intracellular structures. In most bacteria the plasma membrane is overlaid by a continuous covalently linked network of *peptidoglycan* (also called *murein*), consisting of linear chains of polymerized acetamido sugars with short oligopeptide cross-links. In gram-negative bacteria there is also a second *outer membrane* on the outside of the cell envelope, which contains a lipopolysaccharide component in addition to proteins and lipids (Leive, 1973). In eukaryotic cells there are two membranes surrounding mitochondria, as shown in Fig. 16-2, reflecting the fact that mitochondria have probably evolved from a prokaryotic origin.

Higher organisms contain specialized membranes. A well-known example is the myelin sheath surrounding the axon of a nerve cell, as shown in

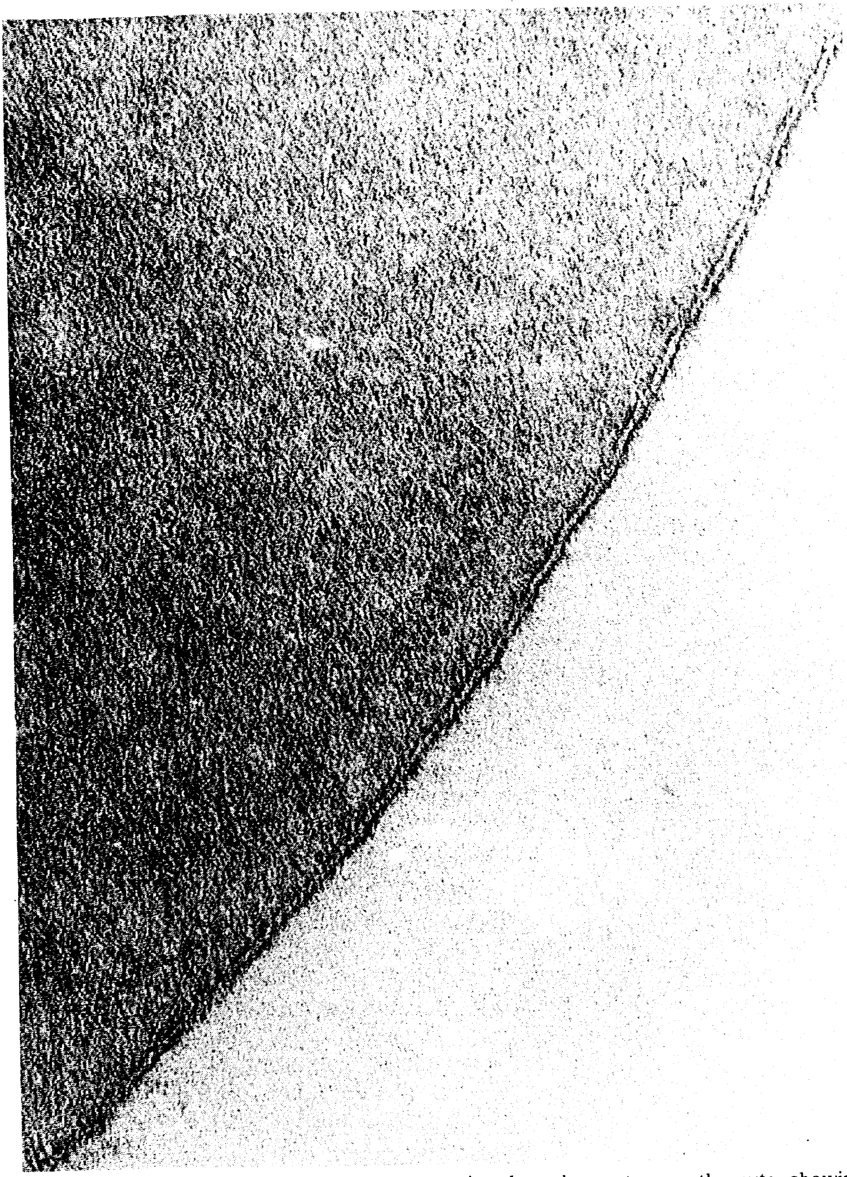

Fig. 16-1. Electron micrograph of a cross section through a mature erythrocyte, showing the cytoplasmic membrane surrounding the cell. The cell has been stained with osmium tetroxide and the membrane appears as two electron dense lines with an intervening space (provided through the courtesy of Dr. J. D. Robertson).

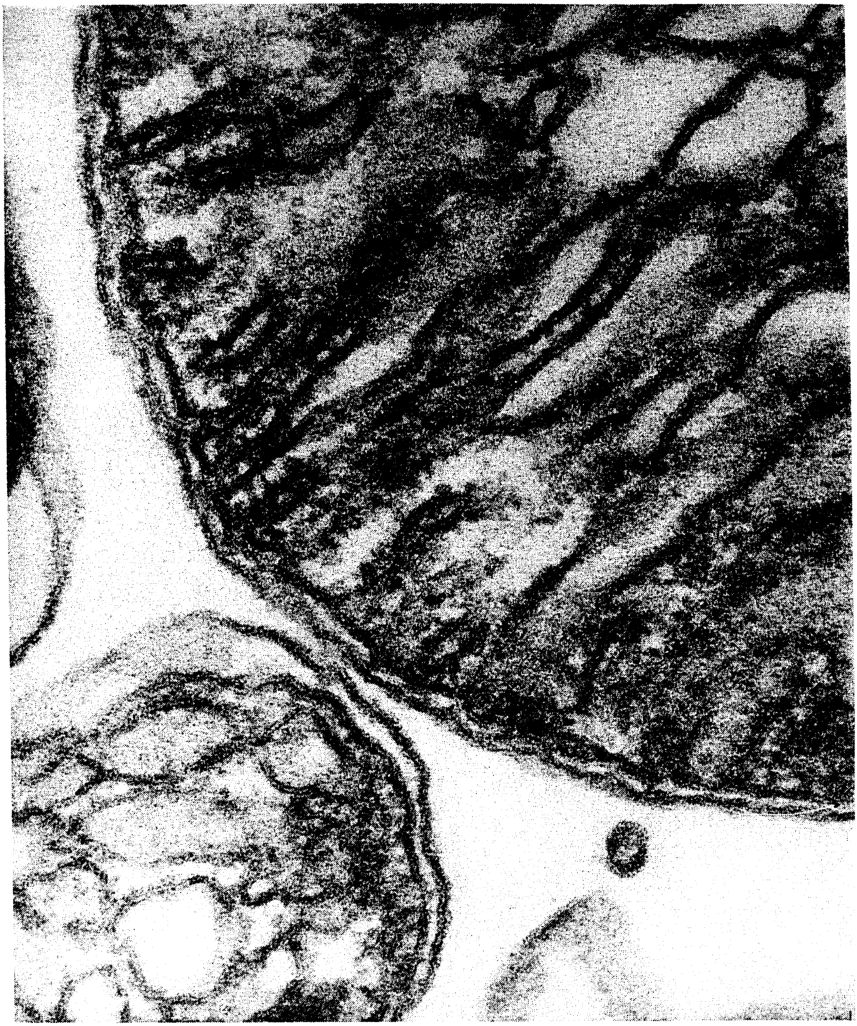

Fig. 16-2. Electron micrograph of a cross section through a mitochondrion. The mitochondrion has a smooth outer membrane and a separate inner membrane with many folds into the interior of the organelle. (Stoeckenius, 1969.) From *Membranes of Mitochondria and Chloroplast* by Professor E. Racker © 1970 by Litton Educational Publishing, Inc. Reprinted by permission of Van Nostrand Reinhold Company.

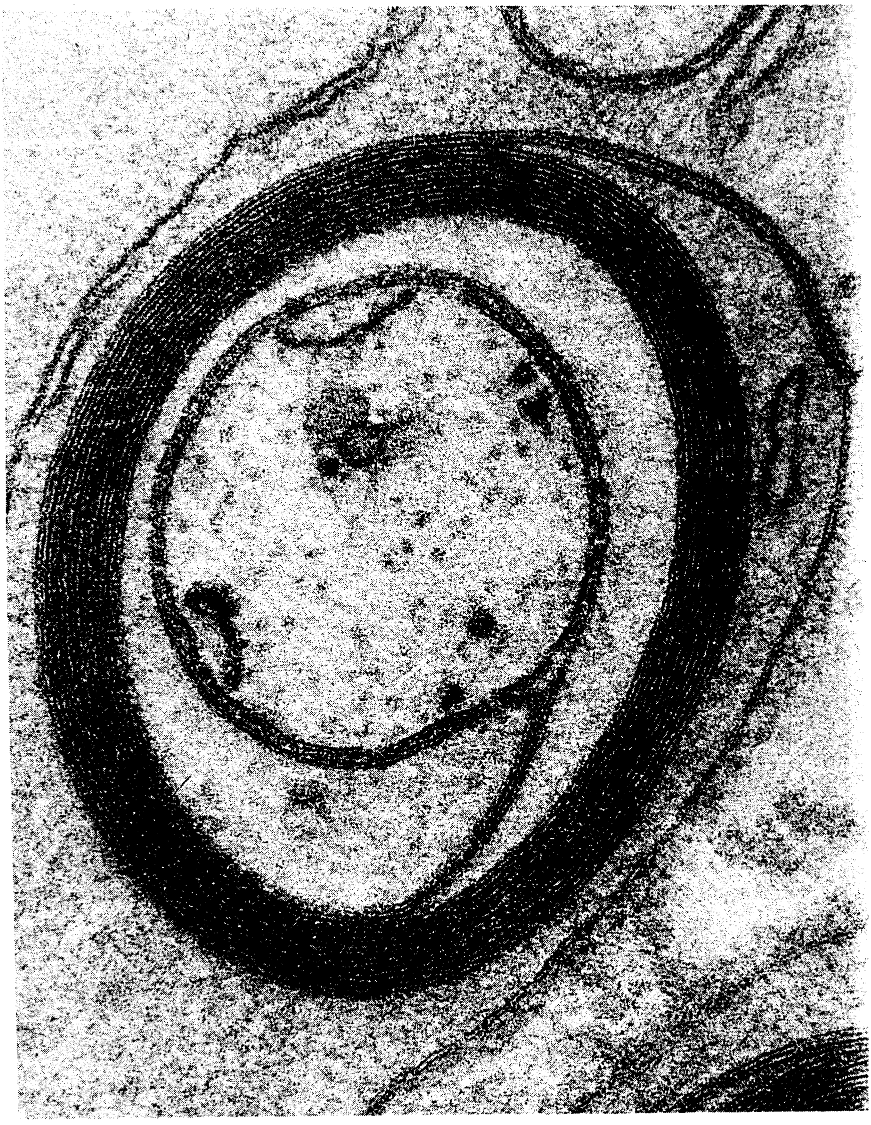

Fig. 16-3. Electron micrograph of the myelin sheath around a nerve axon. The innermost membrane is the axonal membrane, and the surrounding sheath is derived from the plasma membrane of a Schwann cell (provided through the courtesy of Dr. J. D. Robertson).

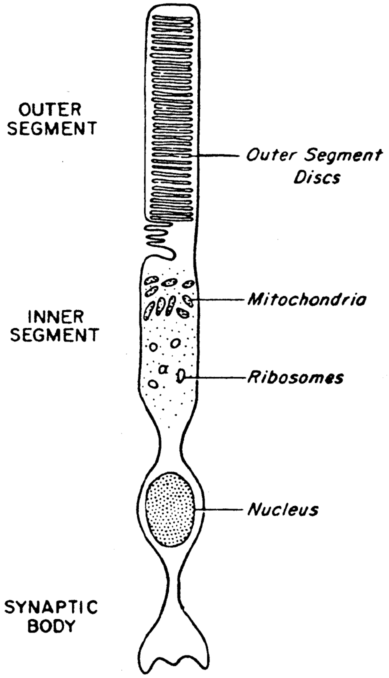

Fig. 16-4. Schematic drawing of a rod photoreceptor cell. The outer segment discs are formed by inward folding of the cytoplasmic membrane, followed by detachment (taken from Heller and Ostwald, 1972).

Fig. 16-3. This structure is formed by extension of the cytoplasmic membrane of a cell called the *Schwann cell* (a glial cell is used by peripheral nerves), which wraps itself in several layers about the axonal membrane. Another example is provided by the stacked discs of the outer segments of retinal rod cells, illustrated by Fig. 16-4. These discs are formed by folding of the outer membrane of the cell and are eventually disconnected from it. A similar folding of the outer membrane occurs in retinal cone cells, but the folds do not become disconnected into discrete discs.

We should note finally that certain viruses have membranous outer shells. They are derived from the cytoplasmic membranes of the host cell, which encapsulates the virus as it leaves the cell. The proteins of the host membranes appear to be generally lost in this process.

OVERALL COMPOSITION

Bacterial inner membranes and all eukaryotic membranes contain proteins and lipids and no other major constituents. Carbohydrate is often

Table 16.1. Analytical Protein and Lipid Content of Several Membranes[a]

	Percent of Dry Weight	
	Protein	Lipid
Myelin	18	79
Human erythrocyte	49	43
Bovine retinal rod	51	49
Mitochondria (outer membrane)	52	48
Acholeplasma laidlawii	58	37
Sarcoplasmic reticulum	67	33
Gram-positive bacteria	75	25
Mitochondria (inner membrane)	76	24

[a] Based on data from Guidotti (1972). Where the figures given do not add up to 100%, the remainder is listed as "carbohydrate." Such carbohydrate would normally not be in free form, but as a constitutive part of glycoproteins or glycolipids.

present, but it is always in combined form as glycolipid or glycoproteins. The proportions of protein and lipid can vary widely, as shown by the analytical data in Table 16-1.

As was pointed out in Chapter 11, the major part of membrane lipid consists of amphiphile molecules with two hydrocarbon tails, except that cardiolipin, which is sometimes present in significant amount, is a dimer of two such molecules, with a covalent link between the head groups. The hydrophilic head groups of these lipids vary considerably between different membranes (Table 11-1), but the hydrocarbon chains are similar. They are always long and contain both saturated and unsaturated hydrocarbons. Cell membranes of eukaryotic organisms may contain up to 25% by weight of cholesterol, but mitochondrial and bacterial membranes contain little or none. The functional significance of compositional differences between different species or between types of membrane within a species is not yet clear, but the almost universal presence of unsaturated acyl chains (cf. p. 110) has an obvious functional advantage because it prevents the formation of ordered crystalline arrays of hydrocarbon chains at physiological temperatures. The membranes are thus always fluid and deformable, and membrane proteins can be inserted into them without disruption of the membrane.

POLYPEPTIDE CHAINS

Membrane proteins, in contrast to membrane lipids, have specialized functions, and a diverse variety of them exists. For analytical purposes, gel electrophoresis in sodium dodecyl sulfate is used to obtain a roster of all the constituent polypeptide chains in the membrane. The basis for this method was discussed in Chapter 14, and it was noted that mobilities on the gel are often, but not always, a measure of the polypeptide molecular weight. Exceptions are somewhat more numerous among membrane proteins than for soluble proteins because membrane proteins have binding sites for phospholipids in the native state that are also capable of binding detergents cooperatively. As a result, the complexes between membrane polypeptides and sodium dodecyl sulfate may not be entirely in the denatured state typical for most polypeptides derived from water-soluble proteins, and their mobilities and other hydrodynamic properties may not depend smoothly on molecular weight. For this reason molecular weight estimates obtained by gel electrophoresis should always be regarded as tentative until confirmed by more direct procedures (Maddy, 1976).

Analytical data obtained by this procedure differ widely from one kind of membrane to another, and major components are often found to be uniquely associated with one particular membrane. The discs in rod outer segments (Fig. 16-4), for example, with a protein content of about 50%, have a single major polypeptide chain constituent, with an apparent molecular weight of about 37,000, which has been identified as the polypeptide chain of the visual receptor protein, rhodopsin. The erythrocyte membrane also contains about 50% protein, but the protein content represents a complex mixture of polypeptide chains, as illustrated by Fig. 16-5. A minimum of 17 polypeptide chains differing in molecular weight must be present, but there are undoubtedly polypeptides that cannot be resolved by this method, that is, some of the discrete bands in Fig. 16-5 probably represent mixtures of different polypeptide chains.

In general the complexity of polypeptide chain composition increases with the diversity of function of the membrane. The two membranes of bacteria, for example, may contain more than 100 distinct polypeptides (Ames and Nikaido, 1976). At the other end of the scale, viral membranes are generally very simple. Sindbis virus, for instance, has only two kinds of polypeptide chain in the entire virus particle, and only one of them is associated with the viral membrane (Strauss et al., 1968).

An important conclusion from all polypeptide chain analyses is that there is no polypeptide constituent common to all membranes, even from

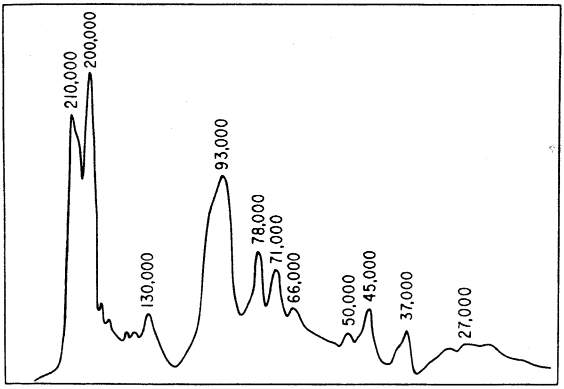

Fig. 16-5. Spectrophotometric scan of the polyacrylamide gel electrophoresis pattern obtained from the mixture of polypeptide chains of a human erythrocyte membrane, dissolved in aqueous sodium dodecyl sulfate. Apparent molecular weight values corresponding to each peak are based on calibration with polypeptide chains of known molecular weight (taken from Trayer et al., 1971).

the same species. There is therefore no evidence to suggest the existence of a "structural" protein which one might imagine as a universal prerequisite for membrane formation. The only common constituents of all membranes are lipids with two hydrocarbon chains, and it seems likely therefore that the spontaneous formation of bilayers by such lipid molecules must also be the underlying mechanism for membrane formation. Structural studies cited below will be seen to demonstrate this unambiguously.

Intrinsic and Extrinsic Proteins

The polypeptide chains seen by analytical gel electrophoresis are derived from proteins which in the native membrane have uniquely folded conformations. As is true for water-soluble proteins, some of the native structures may be oligomeric and may contain more than one kind of polypeptide chain constituent.

It is useful to make an operational distinction between two kinds of membrane-bound proteins. One kind can be removed from the membrane and brought into solution by mild procedures (e.g., changes in ionic strength) that leave the integrity of the membrane as a whole intact. These proteins are presumably associated with the membrane primarily by polar bonds to the membrane surface, and they have been called *peripheral* or *extrinsic* proteins (Green, 1972; Singer and Nicolson, 1972). The second category cannot be released unless the entire membrane is disrupted by use of detergents. They are presumably deeply embedded in the membrane and have been called *integral* or *intrinsic* proteins. Polypeptide chains belonging to extrinsic proteins are readily identified in terms of the loss of specific bands from the analytical gel after the intact membrane has been exposed to appropriate changes in the environment. By this criterion extrinsic proteins constitute about 40% of the total protein of erythrocyte membranes, but in most membranes the fraction of extrinsic proteins is much less.

Biological membranes can be disrupted by nonionic amphiphiles as well as by ionic amphiphiles such as sodium dodecyl sulfate, and intrinsic membrane proteins can often be solubilized in this way without apparent loss of their native structure (Tanford and Reynolds, 1976). This fact is evidence in support of a lipid bilayer as the basic structural framework of a membrane, because it shows that disruption can take place without denaturation of membrane proteins. Since nonionic amphiphiles do not interact at all with most water-soluble proteins, but can form mixed micelles with any other hydrophobic substance, their ability to solubilize intrinsic membrane proteins suggests that these proteins have hydrophobic surface regions that in effect comicellize with membrane lipids in the native state, as will be discussed in more detail in Chapter 17.

STRUCTURAL STUDIES

The idea of a lipid bilayer as a core of the typical membrane was first proposed by Gorter and Grendel (1925), who extracted the lipid from erythrocyte membranes, compressed it at a water–air interface, and found that it occupied a surface area equal to double the external area of the cells from which the lipid was derived.[1] Danielli and Davson (1935) interpreted surface tension measurements (incorrectly, as it turns out) to indicate that lipid head groups were covered by layers of protein, thus accounting for both protein and lipid in terms of a bilayer model. With the advent of electron microscopy, the triple-layered structure shown in Fig.

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[1] As is evident from Fig. 10-2, the surface area of a lipid monolayer depends on the lateral surface pressure at which it is measured. It has recently become possible to estimate the effective pressure in a cell membrane, from which it is concluded that Gorter and Grendel used too low a pressure, and that the actual area occupied by the erythrocyte membrane lipids at the prevailing pressure is less than required for a bilayer. This is of course not an unreasonable result because we now know that membrane proteins are deeply embedded in the bilayer and must occupy part of the surface (Zwaal et al., 1976).

16-1 was found to be an almost ubiquitous image of a membrane. Its similarity to the structures seen in electron micrographs of pure lipid liquid crystals (e.g., Fig. 11-1), the corroborative evidence from X-ray diffraction (see below), and the dimensional compatibility of the image with a bilayer structure led Robertson, who was himself responsible for a major portion of the experimental work, to state the bilayer hypothesis in its most forceful terms (see review by Robertson, 1960). However, the bilayer structure remained controversial until quite recently because the preponderance of protein in most membranes (Table 16-1) suggested that the proteins and not the lipids should dictate the structural organization. Many models were proposed that viewed membranes as formed by self-association of lipid–protein complexes, the forces of association between them being an inherent property of the proteins. The arguments raised in the course of this controversy are reviewed by Stoeckenius and Engelman (1969).

X-ray diffraction studies provide unambiguous evidence for a bilayer structure, and it is worthwhile to describe the data from this source in some detail. X-ray studies of myelin (Fig. 16-3) and retinal rod outer segments (Fig. 16-4) are particularly interesting, for in these two systems nature provides us with a ready-made oriented stack of membranes that may be treated as a one-dimensional crystal. Diffraction from these systems yields several diffraction peaks (Fig. 16-6), which may be analyzed by the methods of X-ray crystallography to yield a low-resolution electron density profile in a direction perpendicular to the plane of the membranes. The phases of the individual peaks may be determined with reasonable certainty by swelling the stack of membranes and assuming that this alters the spacing between membranes, but not the dimensions of individual membranes.

The first X-ray studies of myelin were carried out by Schmitt et al. (1941). The results obtained were similar to those obtained by the same group for hydrated lipids (page 118) and demonstrated the bilayer structure of the membranes convincingly. A spacing of 4.7 Å was obtained in the direction parallel to the bilayer plane, a spacing now known to be characteristic of parallel hydrocarbon chains in bilayers with liquidlike cores. Studies with better resolution have since been made in other laboratories, culminating in the work of Caspar and Kirschner (1971), in which a resolution of 10 Å is attained. The resulting electron density profile is shown in Fig. 16-6, and is remarkably similar to that of pure phospholipid bilayers (Fig. 11-2). The shoulders in the profile at about 15 Å from the bilayer center are ascribable to the presence of cholesterol (26% of the total myelin lipid), and their asymmetric appearance has been considered as evidence that the cholesterol is not symmetrically distributed between the two sides of the membrane.

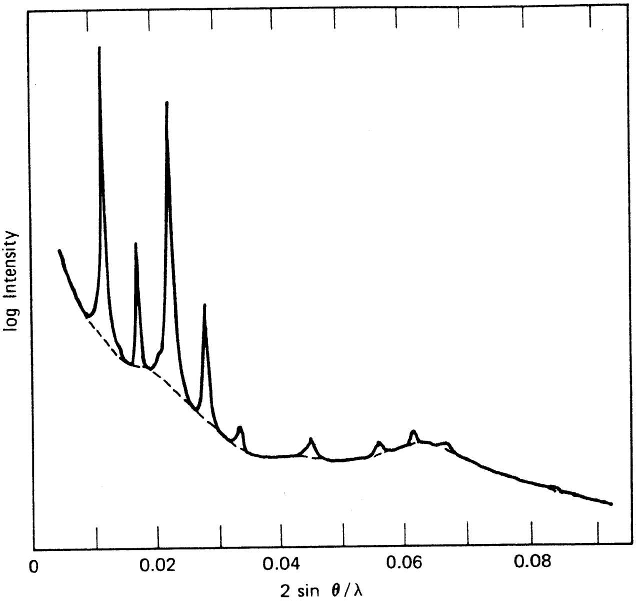

Fig. 16-6. (*a*) Scattered intensity (log scale) as a function of a diffraction angle (θ) for myelin from rabbit sciatic nerves. λ is the wavelength of the X-rays.

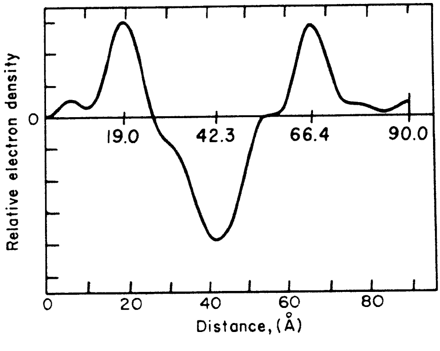

Fig. 16-6. *(Continued)* (*b*) Electron density profile of a single membrane calculated from this diffraction pattern. The solvent density is taken as zero (taken from Caspar and Kirschner, 1971).

The X-ray data for myelin (already known at low resolution in 1941) did not convince proponents of the lipoprotein subunit model of membrane structure because myelin has an atypically low protein content (Table 16-1), and especially as one of the major proteins, myelin basic protein, is an extrinsic protein located on an external surface of the membrane (Eylar, 1976). Similar X-ray data have, however, been obtained for retinal

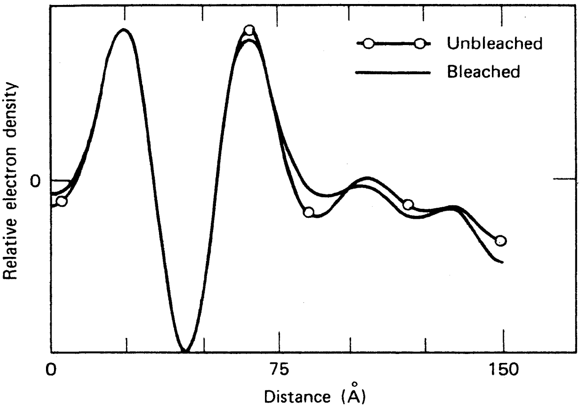

Fig. 16-7. Electron density profile for a single membrane from retinal rod outer segments. Essentially identical results are obtained regardless of whether the photoreceptor protein rhodopsin is bleached or unbleached. The solvent density is taken as zero (taken from Corless, 1972).

rod discs, the membranes of which contain 50% protein, almost all of which represents the photoreceptor protein rhodopsin, which is an intrinsic protein that cannot be removed unless the membrane is first disrupted with detergents. X-ray diffraction measurements of high precision have been made by Blaurock and Wilkins (1969) and by Corless (1972), with essentially identical results. The electron density profile of a single membrane is shown in Fig. 16-7. It consists of two peaks of high electron density, 40 Å apart, and a central trough of electron density below that of water. As previously indicated (page 119), this feature indicates that the center of the membrane is rich in terminal CH_3 groups of hydrocarbon chains. Retinal rod membranes contain little or no cholesterol, and the shoulders in the electron density profile that are seen in myelin are not observed here. The result provides unambiguous evidence that the predominant structural feature of the retinal rod disc membrane is a bilayer similar to that formed by phospholipid alone. The central trough is particularly significant as it can arise only from a region rich in terminal CH_3 groups. Model structures based on protein–lipid structural units appear to be rigorously excluded, since they could not give rise to this feature of the results.

The results leave one major problem unresolved. What is the location of the protein in the membrane? Blaurock and Wilkins (1969) made a careful analysis of the total amount of lipid per unit area in the photoreceptor membrane and concluded that it is insufficient for a pure lipid bilayer with the observed peak-to-peak distance: this distance would have been 22 to 28 Å instead of 40 Å if lipid alone had been responsible for the observed diffraction pattern. This discrepancy is reasonably interpreted in terms of penetration of the bilayer by a substantial portion of the rhodopsin molecule (Blaurock and Wilkins, 1972). Possible evidence for this interpretation is provided by the observation of a diffuse reflection indicating a spacing of about 55 Å in the plane of the bilayer, which could represent the average distance between centers of *mobile* rhodopsin molecules, mobility being conferred on them by the fluidity of the bilayer. A similar spacing had been previously observed by Blasie et al. (1969) in a preparation of centrifuged discs and had been similarly interpreted. Blasie et al. found that the reflection corresponding to this spacing was sharpened when the membranes were first treated with antibody specific for rhodopsin, indicating that combination with the antibody reduces the mobility of the protein. This experiment also indicates that at least a part of the rhodopsin molecule must extend into the aqueous medium external to the membrane.

Wilkins et al. (1971) have carried out X-ray diffraction measurements on random dispersions of erythrocyte membranes and membranes from the microorganism *Acholeplasma laidlawii.* The data obtained from such dispersions are of course much inferior to data obtained from ordered arrays, since a continuous distribution of scattered intensity is obtained with only two or three broad peaks. Nevertheless, the patterns obtained (which were similar to diffraction patterns from dispersions of egg phosphatidyl choline) strongly indicated that a bilayer structure is the dominant one in these membranes also. X-ray diffraction measurements have also demonstrated that membranes present in certain viruses have a bilayer-dominated structure. Sindbis virus is an example. This is a spherical virus bounded by a membrane and containing a glycoprotein external to the membrane. The overall radius is 350 Å, but the outer shell occupied by the glycoprotein is penetrable by solvent. X-ray diffraction measurements of suspensions of these viruses permit evaluation of an electron density distribution function (spherical symmetry assumed) with a resolution of 28 Å (Harrison et al., 1971a). The result obtained is shown in Fig. 16-8. The deep minimum at a radial distance of 232 Å is virtually conclusive evidence for the location of the center of a lipid bilayer at that position. Similar results have been obtained with bacteriophage PM2, another membrane-containing virus (Harrison et al., 1971b).

LOCATION OF PROTEINS AND MEMBRANE ASYMMETRY

The general principles underlying the incorporation of proteins into biological membranes were first formulated by Bretscher (1973). An impor-

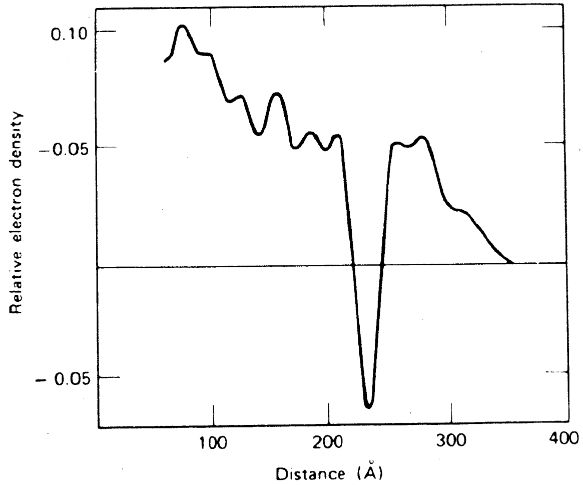

Fig. 16-8. The spherically averaged electron density of whole Sindbis virus, as a function of radial distance from the center. The solvent electron density is taken as zero (taken from Harrison et al., 1971a).

tant point made by him was that cell membranes must differ from pure phospholipid bilayers by being *asymmetric*. The basic reason for this is that one surface of a membrane always faces the cell cytoplasm, where both membrane lipids and proteins are synthesized. The opposite side of a plasma membrane faces the outside of the cell, and the opposite side of an intracellular membrane faces the internal lumen of an intracellular organelle. Except in mitochondria (see below) neither protein nor lipid can be synthesized on the noncytoplasmic side.

Since membrane proteins, like all proteins, contain a high proportion of ionic amino acid side chains, they are unable to exchange between the two sides of a phospholipid bilayer, and this would at first seem to require that all proteins must be confined forever to the cytoplasmic side of the membrane. In fact, however, there is a special mechanism (briefly discussed in Chapter 17) whereby proteins can traverse a bilayer at the time of biosynthesis, and their locations in the membrane remain fixed only after the biosynthetic process is complete. Experiments that have been done to probe accessibility of membrane proteins to reagents added from the outside show that the final locations always remain asymmetric, that is, even if traversal of the membrane occurs, the protein is left uniquely oriented with respect to the site of biosynthesis. In erythrocytes, for example, only two of the large number of constituent polypeptide chains (Fig. 16-5) are accessible to reagents added to the outside surface of the intact cell (Berg, 1969; Phillips and Morrison, 1971; Bretscher, 1971a). All of the proteins can, however, react with suitable reagents after the cell has been lysed to expose the internal surface. Similarly, catalytic or binding sites of proteins involved in physiological function are always uniquely oriented. The catalytic sites of ATP-driven ion pumps, for example, are always on the cytoplasmic side, whereas recognition sites for circulating hormones have to be on the external surface of the cell. Glycosylation of proteins (and lipids) is mediated by enzymes on the noncytoplasmic side, and covalently linked carbohydrate has never been observed to be on the cytoplasmic side of a membrane.

It is of interest to note that Bretscher (1971 b, c) found that the two erythrocyte polypeptides that are exposed on the external surface of the intact cell can be labeled at quite different positions along the chain after lysis of the cell, which indicates that they are exposed on the cytoplasmic side as well, that is, assuming that no reorganization of the membrane occurs during lysis (which is almost certainly true), these two polypeptides must traverse the native membrane from one side to the other. Similar experiments have been done on other membranes, from which it is clear that a substantial number of trans-membrane proteins exist. It is noteworthy that no polypeptides of the erythrocyte membrane were found to be exposed solely on the noncytoplasmic side, and this conclusion appears to apply to most membranes. Membrane proteins may be destined at the time of synthesis to be extrinsic proteins, in which case they invariably remain on the cytoplasmic side. If they are destined to be intrinsic proteins, they either penetrate part of the way into the phospholipid bilayer, or they traverse it all the way to the opposite side, but in either case part of the polypeptide remains on the cytoplasmic side.[2]

It should be mentioned that mitochondria, unlike other intracellular organelles, are capable of protein synthesis because they have evolved from a symbiotic inclusion of a bacterial type of cell within the larger eukaryotic cell. In mitochondrial membranes, therefore, polypeptides are oriented asymmetrically as before, but may be synthesized and inserted from either side. Cytochrome oxidase, a major component of inner mitochondrial membranes, is a well-known example. In yeast, for example, this protein is an oligomer of seven polypeptide chains: three of them originate from inside the mitochondrion and four from the cell cytoplasm (Poyton and Schatz, 1975). How the latter traverse the outer mitochondrial membrane to reach their destination at the inner membrane has not been established.

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[2] An incidental result of experiments related to the external exposure of membrane proteins is to make it easier to understand how membrane structure can be determined by the lipid component rather than the often more abundant protein component. Parts of all membrane proteins extend into the adjacent fluid beyond the bilayer portion of the membrane, and the amount of protein actually within the domain of the bilayer structure is usually far less than the *analytical* protein content.

Direct evidence on membrane asymmetry comes from experiments using freeze fracture electron microscopy. In this technique a specimen in aqueous suspension is frozen and then fractured by a blow from a microtome knife. The fracture will tend to seek out planes containing the weakest intermolecular bonds, which, in a system containing lipid bilayers, would be the central region of the bilayer in which the ends of the lipid hydrocarbon chains are concentrated.[3] The interior of the bilayer is thereby exposed and a replica of its morphology can be obtained by evaporation of a film of metal onto it. When these replicas are examined in the electron microscope, smooth fracture planes are obtained from pure lipid systems and also from myelin (Branton, 1967). Most other membranes produce rough surfaces, suggesting that the fracture has occurred around particulate obstacles, and the majority of the observed bumps is nearly always associated with the half of the bilayer facing the cytoplasm, as in the example shown in Fig. 16-9. Although it cannot be established that the particles observed in these pictures represent actual outlines of protein molecules or clusters of protein molecules, they do represent severe distortions of the bilayer interior for which proteins penetrating into the bilayer must be directly or indirectly responsible.

Experiments that probe accessibility of lipid head groups show that phospholipids are also asymmetrically distributed in native membranes. For example, in erythrocyte membranes (see Table 11-1 for composition), the lipids with choline head groups, phosphatidylcholine and sphingomyelin, are more abundant in the external half of the bilayer, whereas phosphatidylethanolamine and phosphatidylserine are more abundant on the cytoplasmic side. The reasons for this are not yet clear and may be related to events occurring at the time of lipid biosynthesis (Rothman and Kennedy, 1977). This and other aspects of membrane asymmetry are discussed in greater depth than has been possible here in a review by Rothman and Lenard (1977).

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[3] Fracture of the frozen specimen is performed in a vacuum, so that freshly exposed surfaces consist of *unbonded* molecular surfaces. The situation is completely different from disruption of a bilayer in an aqueous medium, where freshly created surfaces would be exposed to water, so that the hydrophobic areas would be the least likely to be exposed.

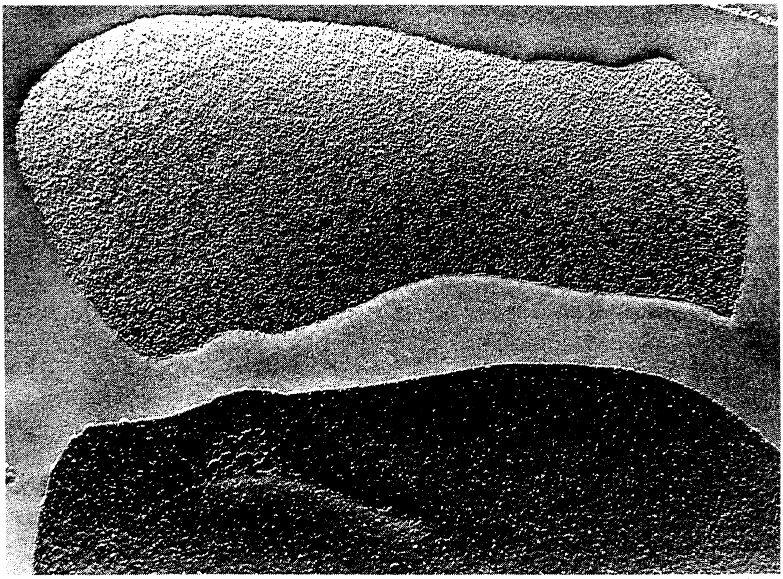

Fig. 16-9. Freeze fracture electron micrograph of an erythrocyte membrane. The upper part of the photograph represents the side of the membrane facing the cell interior and contains many more protuberances than the lower part of the photograph, which is the outfacing side of the membrane of an adjacent cell. (Obtained through the courtesy of Knute A. Fisher, Department of Botany, University of California, Berkeley.)

CYTOSKELETON

One of the functions of a membrane is to define the size and shape of the compartment it encloses, and the cytoplasmic membrane in particular defines the size and shape of a whole cell. The molecular organization of phospholipids into bilayers cannot account for the specific sizes and shapes that cells are known to adopt, and this aspect of membrane structure must be determined by an external "skeleton." In bacteria the skeleton is created by the peptidoglycan layer in the cell wall outside the plasma membrane, mentioned earlier in this chapter. In eukaryotic cells, however, the skeleton is formed by proteins on the cytoplasmic side (hence the name *cytoskeleton*), the existence of which can be demonstrated by appropriate microscopic techniques (e.g., Buckley and Porter, 1967; Osborn et al., 1978). These skeletal proteins provide a relatively rigid framework which determines the extent and shape of the overlying membrane. No penetration of the membrane by the cytoskeleton is necessary, and the skeletal proteins are in fact among the extrinsic membrane proteins that can be released from membrane preparations without disruption of the membrane bilayer or its constitutent intrinsic proteins. Many of the cytoskeletal proteins are closely related to muscle proteins, indicating that the cytoskeleton can often be considered to be a "cytomuscular" structure, which, though rigid, can contract to permit changes in cell shape. The budding out and pinching off of small vesicles from the membrane in pinocytosis and exocytosis and the translational movement of cells (e.g., by extension of pseudopodia) almost certainly involve the cytoskeleton, but the exact mechanism is not yet understood. There are several good reviews on the subject (Singer, 1974; Goldman et al., 1976; Clarke and Spudich, 1977).

In the erythrocyte, the cytoskeletal proteins represent an unusually high proportion of the total protein content. The two polypeptides of highest molecular weight seen in the analytical gel of Fig. 16-5 and the polypeptide with apparent molecular weight of 45,000 are the main constituents of the cytoskeleton. The high molecular weight peptides are combined into a fibrous protein called *spectrin*, which has some resemblance to muscle myosin, whereas the smaller peptide is among the proteins known as nonmuscle actins, which appear to be universal constituents of all eukaryotic cells (Clarke and Spudich, 1977). Spectrin appears to be unique to erythrocytes, but related proteins occur in many other cell types (e.g., Wang et al., 1975).

MOLECULAR MOTION

Measurements related to molecular motion of phospholipid molecules, of the kind described for pure phospholipid bilayers in Chapter 12, have been applied to biological membranes as well, and similar results have been obtained to some extent. Hubbell and McConnell (1969) have shown, for example, that spin labels inserted into axonal membranes from the walking-leg nerve of the lobster display rapid anisotropic motion similar to that observed when these labels are inserted into phospholipid vesicles, and they have observed a similar increase in local fluidity as the label is moved along the hydrocarbon chain from the head group toward the terminal methyl group. Qualitatively similar results were obtained for erythrocyte membranes, but the overall fluidity in the hydrophobic core was found to be much lower. Similar restrictions on the mobility of hydrocarbon chains in erythrocyte membranes have also been observed by nmr measurements (Chapman et al., 1969). In another paper, Scandella et al. (1972) have shown that spin-labeled phospholipid molecules are capable of lateral diffusion in sarcoplasmic reticulum membranes, and that the rate of diffusion is about as fast as in a pure phospholipid bilayer. As in pure phospholipid bilayers, the rate of transverse diffusion ("flip-flop") is generally slow (Rousselet et al., 1976a,b), but faster than normal trans-membrane lipid exchange has been reported in one study of an excitable membrane (McNamee and McConnell, 1973).

The high content of unsaturated acyl chains in membrane phospholipids (p. 110) normally prevents formation of lipid crystalline phases when the temperature is lowered, and the phase transitions described for pure phospholipids in Chapter 12 are generally not observed in natural membranes and are not thought to play a role in biological processes. It is possible to observe such phase transitions in the membranes of living organisms in the laboratory in exceptional situations where it is possible to manipulate the hydrocarbon chain composition of the lipids. Such manipulation is possible in the prokaryotic organism *Acholeplasma laidlawii* and in appropriate mutants of *Escherichia coli* that are deficient in the ability to synthesize fatty acids and thus require externally added fatty acids for growth. An abnormally high content of saturated acyl chains can be introduced in this way and part of the membrane lipid can be converted to an ordered phase without serious impairment of life processes. What probably happens is that patches of ordered lipid separate from the fluid regions of the membrane (p. 134) and the physiological function of the membrane is confined to the fluid regions. Data on phase transitions in these systems are reviewed by Overath and Tilo (1978).

Proteins, as well as lipids, often have considerable freedom of motion in the plane of the membrane in which they are embedded. Evidence for rotational freedom comes, for example, from observations on the dichroism associated with absorption of polarized light by the photoreceptor protein rhodopsin. The chromophore of this protein, in the intact membrane, has been shown by these studies to be in a state of rapid rotational motion (Brown, 1972; Cone, 1972). If the membrane-bound protein is immobilized by cross-linking, the rotational motion is eliminated, indicating that it is the protein molecule itself, or a major part of it, that is responsible for the mobility in the native state.

Evidence for translational mobility is provided by the work of Frye and Edidin (1970) who fused cells from tissue culture lines of mouse and human origin, each bearing surface proteins with distinct antigenic determinants. The movement of the antigenic sites could be followed by use of fluorescent antibodies, and it was observed that both antigens became randomly distributed over the fused hybrid cells within about 40 min. Another example is provided by the microsomal protein cytochrome b_5. This protein can be combined with vesicular preparations of microsomal lipids, and the product obtained in this way cannot be distinguished from endogenous membrane-bound protein. The amount of protein in the vesicular preparation can be varied over a wide range, and the effect of altering the amount on the rate of reaction with the specific enzyme, cytochrome b_5 reductase, strongly suggests that both cytochrome b_5 and the reductase are mobile in the membrane and react with each other by random collision (Rogers and Strittmatter, 1975). There is also direct evidence that rhodopsin molecules have translational as well as rotational mobility (Poo and Cone, 1974), as suggested by the X-ray data cited earlier.

The Fluid Mosaic Model

Results of this kind led Singer and Nicolson (1972) to formulate a general model for membrane structure, known as the "fluid mosaic" model, which depicts membranes as two-dimensional solutions of integral proteins in a fluid solvent created by the lipid bilayer. This model has been useful as a starting point for more detailed studies of membrane organization, and has also served to dispel the last remnants of the notion that membrane organization might be based on association between lipoprotein particles. As a general model it is, however, an oversimplification, for restrictions on freedom of motion exist to varying degrees in virtually all membranes, and both lipids and proteins are affected, as described in the following paragraphs.

Boundary Lipid

The insertion of a protein into a phospholipid bilayer creates an interfacial region where lipid molecules cannot have the same overall environment as they do in a pure phospholipid bilayer. The environmental difference is recognized by several different experimental techniques, which show that the general similarity between phospholipid molecules in pure lipid bilayers and in protein-containing membranes applies only to part of the membrane lipid. One of the earliest observations of this kind was the finding by Lenard and Singer (1968) that only 70% of the phospholipid head groups of erythrocyte membranes are susceptible to the action of the hydrolytic enzyme phospholipase C. Similar evidence comes from calorimetric measurements of thermal transitions in *Acholeplasma* membranes containing a high proportion of saturated fatty acids (p. 110), so that the order–disorder lipid phase transition can be observed (Steim et al., 1969). The transition was found to occur in whole membranes at the same temperature as in protein-free dispersions of the lipids alone, but the amount of heat absorbed in the transition was decreased by 25% when protein was present, suggesting that this fraction of the lipid does not participate in the transition. More direct evidence comes from esr measurements of spin-labeled phospholipids and other amphiphiles (Jost et al., 1973; Dehlinger et al., 1974). A fraction of the probe molecules is found not to contribute to the normal rapid motion of the labeled hydrocarbon chains, and the number of molecules in this fraction is proportional to the protein content in situations where the protein content can be varied.

It is not necessary to postulate tight binding of lipid to protein to account for these results, since interference with motional freedom may simply represent a "wall effect" (Marčelja, 1976), for example, interference by the mere presence of a large rigid protein molecule with *trans-gauche* transitions of the hydrocarbon chain. A recent study of Oldfield et al. (1978), employing deuterium magnetic resonance, suggests in fact that lipid molecules at the boundary of an intramembranous protein are not immobilized, but only slowed in their rate of motion. No loss of mobility ascribable to the presence of protein could be detected in the dmr experiments, and the authors suggested that the discrepancy with the esr results arises from the difference in the spectroscopic relaxation times between the two methods (p. 49): esr data are on a time scale of 10^{-8} sec, and molecular motions not averaged out in a period of 10^{-8} sec will not contribute to the sharp spectral signal characteristic of average motion. The time resolution of dmr measurements, on the other hand, is of order 10^{-5} sec, so that a simple slowing down of hydrocarbon chain fluctuations from a frequency $>10^8$/sec to 10^5 to 10^7/sec would cause the chains to appear immobilized by the criterion of esr, but not by the criterion of dmr.

Restrictions on Protein Mobility

As noted above, membrane-bound proteins can often diffuse freely in the plane of a membrane, but experimentally measured diffusion rates vary widely (e.g., Schlessinger et al., 1976), and in some membranes there appear to be barriers that prevent diffusion of proteins altogether. Synaptic endings of nerves, for example, have different membrane-localized functions and contain different membrane proteins from those along the major portion of the axon, in spite of the fact that both regions are served by the same continuous plasma membrane. A similar situation (within a distance of only about 50 μm) occurs in the photoreceptor cell shown in Fig. 16-4: the transport functions located in the plasma membrane are quite distinct in the outer and inner segments and the constituent membrane proteins in the two segments are therefore presumably unable to mix by diffusion.

Such immobilization of intrinsic membrane proteins is generally thought to involve the extrinsic cytoskeleton, but the mechanism of interaction between extrinsic and intrinsic proteins is still in question. The problem is being investigated most thoroughly in the erythrocyte membrane, especially by Branton and co-workers, who have approached the problem by studying the aggregation of the intramembranous particles seen in freeze fracture electron microscopy (Fig. 16-9). These particles are a manifestation of the existence of trans-membrane proteins (in the erythrocyte, primarily the anion transport protein and glycophorin), and the relative ease with which aggregation can be induced is thus a measure of the freedom of motion of these proteins. The results show that the proteins are highly immobilized when the cytoskeleton is present, but they suggest that this is simply the result of entanglement between the trans-membrane proteins and the spectrin-actin meshwork, and that direct protein–protein interactions are not involved (e.g., Elgsaeter et al., 1976). However, there is evidence that specific protein–protein interaction does play a role in the attachment of spectrin to the membrane surface, although the intrinsic protein that carries the presumptive attachment site is not one of the principal trans-membrane proteins (Bennet and Branton, 1977; Bennett and Stenbuck, 1979).

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SEVENTEEN

MEMBRANE PROTEINS

Membrane proteins perform some of the most important basic functions of living organisms. Even in the simplest unicellular organisms the intrinsic proteins of the plasma membrane are essential for entry of nutrients into the cell and for maintenance of osmotic equilibrium between the inside of the cell and its environment. In higher forms of life the proteins of the plasma membranes control all communication between cells, for example, by transmission of nerve impulses or by release and recognition of hormones. In addition, the proteins in the membranes of intracellular organelles enormously enlarge the variety and precision of processes occurring within individual cells. Because of their functional importance, these proteins are currently the subject of intensive biochemical research, comprehensive discussion of which is beyond the scope of this book. This chapter is therefore confined to a summary of the present still rudimentary knowledge of those aspects of membrane protein chemistry that are directly related to the hydrophobic effect: features of the molecular structure that are responsible for insertion into membranes, and the mode of interaction between protein and lipid that keeps them there. Only *intrinsic* membrane proteins will be discussed. As noted earlier (p. 188), *extrinsic* proteins interact with membranes primarily by polar bonds to the membrane surface. Hydrophobic interactions may play a role in some instances (e.g., myelin basic protein; Eylar, 1976), but it is a minor role and at present not well defined for any specific extrinsic protein.

Because of the large number and varied function of membrane proteins, it is unlikely that a single model for interaction with a lipid bilayer will apply to all of them. The limited factual data available at present suggest that the interaction may for many proteins be almost entirely hydrophobic. Where this is true, the preferred folded conformation of the protein molecule, instead of having a uniform and predominantly hydrophilic surface such as water-soluble proteins have, may consist of spatially distinct domains, at least one of which has a predominantly hydrophobic surface. The overall structure would then be amphiphilic and the thermodynamically favored location of the protein would be at an interface where a hydrophobic domain could reside in a hydrocarbon medium while the hydrophilic domains would remain in the adjacent aqueous medium. If the protein is intended to penetrate the membrane from only one side, a hydrophobic domain might be created by a loop in a polypeptide chain, which in the folded structure extends away from the hydrophilic part of the molecule. If the protein is intended to traverse the membrane from one side to the other, the hydrophobic domain would have to be in the middle of the folded structure, with hydrophilic domains on both sides.

Evidence supporting such a simple mode of protein–lipid association exists for a few proteins, as the following pages will show. Only future research can show how general this picture is.

STRUCTURAL INFORMATION

Complete amino acid sequences are available at the time this is being written for only three intrinsic membrane proteins, all of them small and containing only a single polypeptide chain. All three polypeptides have a normal overall amino acid composition with respect to the content of residues with hydrophilic and hydrophobic side chains. However, the distribution of the amino acid residues along the polypeptide chain is abnormal, and each of the proteins contains a long sequence segment of mostly hydrophobic residues, uninterrupted by ionic or strongly hydrophilic residues that would be thermodynamically unstable if withdrawn from an aqueous environment. A portion of the sequence of one of these proteins, the M,N-glycoprotein from the erythrocyte membrane (often called *glycophorin*), is shown in Fig. 17-1. The hydrophobic segment is seen to have a length of 23 amino acids, and its only hydrophilic moieties are the four OH groups located on serine, threonine, and tyrosine residues. The free energy lost by withdrawing these OH groups from contact with water would be at most 20 kcal/mol (p. 17), and it could be considerably less if hydrogen bonds can be formed between the OH groups in the membrane-bound structure. The free energy favoring incorporation in a hydrocarbon medium contributed by the hydrophobic amino acids is close to 30 kcal/mol. The coat protein of the filamentous bacteriophage fd (which is embedded as an intrinsic membrane protein in the plasma

70 75 80
-Glu-Pro-Glu-Ile-Thr-Leu-Ile-Ile-Phe-Gly-Val-Met-

85 90
Ala-Gly-Val-Ile-Gly-Thr-Ile-Leu-Leu-Ile-Ser-Tyr-

95 100 105
Gly-Ile-Arg-Arg-Leu-Ile-Lys-Lys-Ser-Pro-Ser-Asp-

Fig. 17-1. Part of the amino acid sequence of the M,N-glycoprotein (glycophorin A) from human erythrocyte membranes, from Tomita et al. (1978). The protein contains 55% carbohydrate, which is attached at various positions along the amino-terminal portion of the polypeptide. The part of the sequence shown contains the 23-residue predominantly hydrophobic segment (underlined), which is the presumed site for association with lipid. The total length of the polypeptide is 131 residues.

membrane of its host bacterium during phage morphogenesis) has a similar long stretch of hydrophobic amino acids (Nakashima and Konigsberg, 1974). Cytochrome b_5, a microsomal membrane protein that penetrates the membrane only part way from the cytoplasmic side (see below), has a somewhat shorter hydrophobic segment containing 15 residues (Ozols and Gerard, 1977; Fleming et al., 1978).

Glycophorin is one of the erythrocyte proteins that spans the membrane (p. 195), and the amino acid sequence clearly lends itself to the formation of a hydrophobic domain bounded on both sides by hydrophilic domains. If the hydrophobic sequence segment were to be folded into an α-helical conformation, it would form a narrow cylinder with a hydrophobic surface of sufficient length to traverse the normal 30 Å thickness of the hydrophobic core of a bilayer, as shown in Fig. 17-2*a*. There would be no exposed peptide groups because all such groups are internally hydrogen bonded in the helical structure. If the hydrophobic sequence were in an extended β-conformation, only nine residues would provide sufficient length to traverse the bilayer core (Fig. 17-2*b*). However, since hydrogen bonds can be formed in a β-conformation only if they extend laterally from the backbone, several parallel strands would be necessary to form a structure without unbonded peptide groups in the hydrophobic domain. Reversals in the direction of a single chain could occur as in Fig. 17-2*c*, so that more than one strand could originate from a single polypeptide, but 23 residues would not be enough to form a stable overall structure. If the hydrophobic segment of glycophorin has the β-conformation, the protein would probably have to traverse the membrane as a dimer or higher oligomer. (There is some uncertainty because the bilayer is liquid and could conceivably be compressed in the vicinity of an inserted protein to accommodate itself to a hydrophobic domain less than 30 Å in length.)

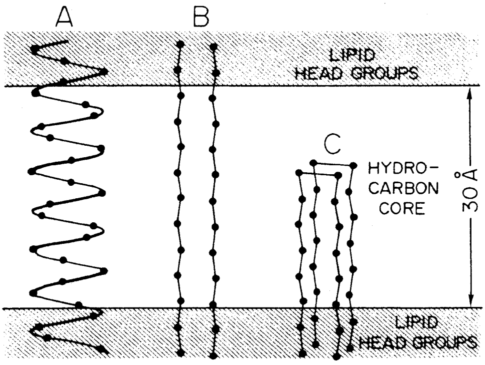

Fig. 17-2. Simple possibilities for the penetration or traversal of the hydrophobic core of a phospholipid bilayer by intrinsic membrane proteins. Filled circles represent α-carbon atoms of the polypeptide backbone, drawn at correctly scaled separations of an α-helix (*A*), an anti-parallel β-structure (*B*), and a β-structure with 180° turn (*C*). Four or more strands are required to generate a complete set of hydrogen bonds between extended chains in the β-conformation; for as few as four strands (as in *C*) the chain backbone would have to be severely twisted. Hydrophobic side chains attached to the α-carbon atoms provide the thermodynamic driving force for location in the hydrophobic core.

The actual state of aggregation and chain conformation of glycophorin in the erythrocyte membrane are not known. There is, however, good evidence that the hydrophobic segment of the fd coat protein has the β-structure when bound to phospholipid bilayers in reconstituted systems (Chamberlain et al., 1978). This protein is also known to be in a dimeric state when associated with amphiphiles, so that a multistrand structure is a good possibility. Whether the coat protein spans the membrane in its membrane-associated state is uncertain.

The fact that cytochrome b_5 consists of separate hydrophilic and hydrophobic domains was established on the basis of limited proteolysis several years before the sequence was determined: proteolysis of the native membrane separates cytochrome b_5 into a large globular water-soluble fragment, which retains the biological function of the protein, and a carboxy-terminal segment of about 40 residues containing the site for association with the phospholipid bilayer (Ito and Sato, 1968; Spatz and Strittmatter, 1971), as shown schematically in Fig. 17-3. Since the protein lies entirely on one side of the membrane, it is not known how far the 15-residue hydrophobic segment has to penetrate into the bilayer core.

Another protein with a domain structure like that of cytochrome b_5 is the enzyme cytochrome b_5 reductase, which interacts with cytochrome b_5 in the microsomal membrane (Spatz and Strittmatter, 1973). Limited

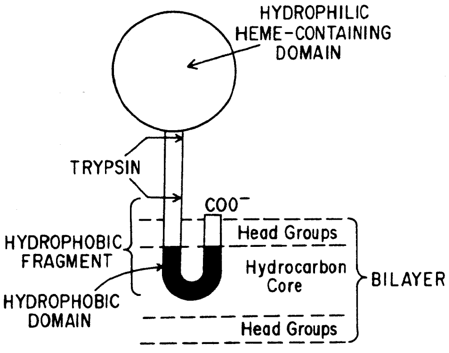

Fig. 17-3. Schematic representation of cytochrome b_5 in a phospholipid bilayer, based on data derived from limited proteolysis and from dimensional studies in detergent solutions, where the protein is always found to be in the monomeric state (Visser et al., 1975). Subsequent sequence data have demonstrated the existence of a 15-residue hydrophobic segment, located 8 residues from the carboxy-terminal end (Ozols and Gerard, 1977; Fleming et al., 1978).

proteolysis studies of other membrane proteins show, however, that this is by no means a general structural feature. Several proteins have been found to have specific sites where clean scission of the polypeptide chain into two or more unique fragments can occur, but, in contrast to cytochrome b_5, all of the fragments prove to behave like intrinsic membrane proteins and remain associated with the phospholipid bilayer unless detergents are used to disrupt the bilayer. Examples of proteins in this category are rhodopsin (e.g., Fung and Hubbell, 1978), the calcium pump protein from sarcoplasmic reticulum (e.g., Rizzolo et al., 1976), and the anion transport protein of the erythrocyte (Drickamer, 1977; Steck et al., 1978). All three of these proteins are known to span the membrane. Since proteolysis occurs in the aqueous medium outside the physical domain of the bilayer, the polypeptide chains must contain two or more independent segments in the hydrophobic core of the bilayer, with hydrophilic loops between them. The length of polypeptide chain incorporated into intramembranous segments is not known because proteolysis data yield only the length between the externally located peptide bonds that are susceptible to hydrolysis. As the subsequent discussion will show, it is possible that long intramembranous segments could form structures in the hydrophobic core that do not require a totally hydrophobic amino acid sequence.

Intrinsic membrane proteins require phospholipids or suitable amphiphilic analogs to retain their native structure, and it would seem to be a formidable task to obtain them in crystalline form for the determination of precise structures by X-ray diffraction. It has, however, been possible to obtain reliable information (at a resolution of about 7 Å) about the three-dimensional structure of one membrane protein, bacteriorhodopsin, from the organism *Halobacter halobium*. This protein fortuitously exists in the

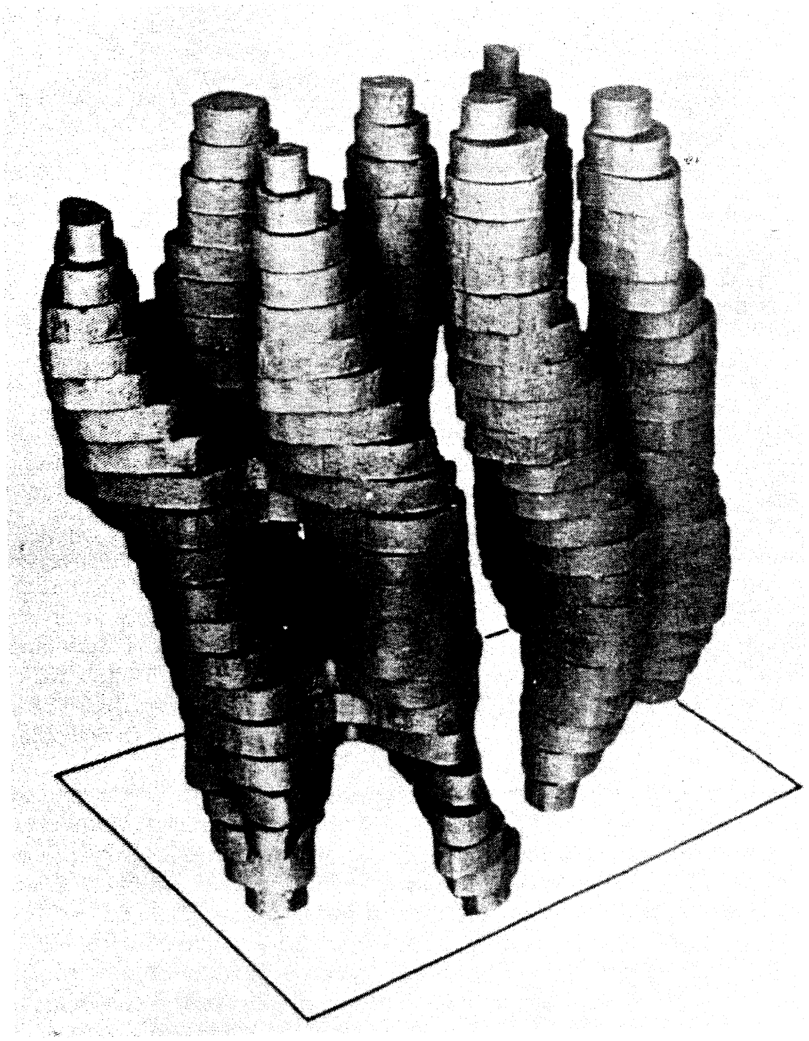

Fig. 17-4. Model of a single molecule of bacteriorhodopsin in the purple membrane, derived from structural studies at 7 Å resolution. The model shows the parts of the molecule that extend through the membrane; external links, comprising about 30% of the molecular mass, are not shown (taken from Henderson and Unwin, 1975).

membrane in a two-dimensional crystalline array, and this has made possible a determination of a part of the structure by a combination of transmission electron microscopy and electron diffraction (Henderson and Unwin, 1975). The protein consists of a single polypeptide chain of molecular weight 25,000 and the part of the structure that has been determined comprises about 70% of the polypeptide. It consists of seven short side-by-side rods, as shown in Fig. 17-4, which almost certainly represent polypeptide chain segments in an α-helical conformation. When the electron density of the solvent is varied, it has no effect on the density contrast between these helical regions and their environment, so that the helices presumably span the membrane, and are joined to each other on both sides by hydrophilic polypeptide segments.

An important aspect of the bacteriorhodopsin structure is that the overall amino acid composition of the protein is not strikingly unusual. The external hydrophilic links in the structure are not long enough to contain all the hydrophilic amino acid residues of the protein, and it is therefore not possible that all or nearly all of the surface of the transmembrane helices can be devoid of ionic or strongly polar amino acid side chains. The complete amino acid sequence of the protein was published while this chapter was in press (Ovchinnikov, 1979). Speculative arrangements of the polypeptide chain in the membrane were proposed, but it is too early to identify the interactions that permit the presence of hydrophilic amino acid side chains in the plane of the phospholipid bilayer. There has not been time to see whether the sequence is compatible with the kind of possibility suggested below in Fig. 17-5.

TRANSPORT ACROSS MEMBRANES

The physiological function of many membrane proteins is to permit controlled transport of ions or other hydrophilic solutes across the membrane. The bilayer-associated regions of these proteins must be more complex than the simple hydrophobic polypeptide domains described above for glycophorin or cytochrome b_5. It would seem essential that the bilayer-associated domain must contain (or be able to create on demand) a hydrophilic "channel" through which the solutes can pass. Bacteriorhodopsin functions in the purple membrane as a light-driven proton pump and is therefore one of the proteins in this category: the probable existence of hydrophilic amino acid residues in its intramembranous domain is therefore consistent with its physiological function.

Several antibiotics are known to form conductance channels through phospholipid bilayers. One of them, gramicidin A, is an oligopeptide containing 15 amino acid residues, alternately in L and D configurations. All of the amino acid side chains are hydrophobic, and by virture of the alternation in optical configuration the oligopeptide is capable of forming helical structures with a central pore lined by hydrogen-bonded peptide groups, with side chains extending out from the surface. The probable structure (Urry et al., 1971; Hladky and Haydon, 1972) consists of two molecules of gramicidin, approaching the bilayer center in opposite directions from the two sides of the membrane. The helical conformation has 6.3 residues per turn, and the central pore has a dimeter of 4 Å, sufficient to allow passage of water and small cations.

Protein polypeptides have all amino acids in the L-configuration, and a single polypeptide segment could not form a helical structure with a central cavity unless every alternate amino acid residue were glycine. Channel-containing structures can, however, be formed by participation of several polypeptide segments, and one possible structure of this type was suggested by Inouye (1974) in a somewhat different context. The structure consists of six parallel twisted α-helices, as illustrated by Fig. 17-5. Each helix contains both hydrophobic and hydrophilic amino acid side chains, periodically arranged so that all side chains facing the outside are hydrophobic, while those on the inside are hydrophilic. The individual helical segments could originate from the separate polypeptide chains of an oligomeric structure, or they could be parts of a single polypeptide chain that crosses the membrane several times, as in Fig. 17-4. In the model depicted by Fig. 17-5, the central pore of the structure is wide enough to represent an actual solvent-filled channel, but the model is intended to be only schematic. The operational definition of the term "channel" includes much narrower passages in which transport might occur by mechanisms other than free diffusion through a liquid solvent column; polar groups in the passage would, however, be required for any mechanism.

There is obvious similarity between the model of Fig. 17-5 and the structure of bacteriorhodopsin, but a narrow pore within the bacteriorhodopsin structure would not be detectable at the limited resolution

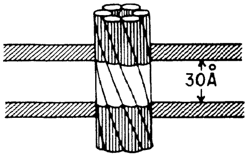

Fig. 17-5. Hypothetical model for formation of a permeability channel across a phospholipid bilayer, based on a proposal by Inouye (1974) for the structure of the major cell wall protein of *Escherichia coli*. The twisted cylinders are helical segments of polypeptide chains. Unshaded surfaces represent location of hydrophobic amino acid side chains, and shaded surfaces are hydrophilic.

of the data. In the overall structure of the purple membrane (Henderson and Unwin, 1975) three protein molecules with the structure illustrated by Fig. 17-4 are arranged in a circle around a central hole with a diameter of about 20 Å. This hole has, however, been found to be inaccessible to solvent when effects of changes in solvent electron density on diffraction intensity are investigated. This and other evidence indicates that the 20 Å cavity is filled with lipid.

BINDING OF SIMPLE HYDROPHOBIC LIGANDS

Binding with simple hydrophobic ligands could serve to define the binding regions of membrane proteins, as they have done to some extent for serum lipoproteins (Chapter 15). If the mode of association of protein with lipid can be thought of as analogous to mixed micelle formation, as suggested by the preceding discussion, then the protein should also be capable of forming mixed micelles with simple amphiphiles. In binding studies this would lead to "micellar binding," as defined in Chapter 14, that is, cooperative association between the protein and the amphiphile, in which the amount of bound amphiphile would be about the same as the amount contained in a protein-free micelle. Only scattered data are available so far, insufficient for a general conclusion. One of the difficulties in carrying out binding studies is that intrinsic membrane proteins are usually insoluble in aqueous media if their hydrophobic binding regions are exposed without any ligand bound to them.

Cytochrone b_5 (Fig. 17-3) is soluble in aqueous media in an aggregated state (Calabro et al., 1976). Studies of the binding of deoxycholate[1] and of the nonionic detergent Triton X-100 to this protein support the existence of a nonspecific hydrophobic binding region at one end of the molecule (Robinson and Tanford, 1975). Little or no binding is observed below the cmc of the amphiphiles, but cooperative association occurs near the cmc, the maximal amount bound in each case corresponding approximately to the amount of amphiphile contained in a micelle in the absence of protein, that is, binding satisfies the criterion for "micellar binding," in contrast to what was observed with the A-I polypeptide of high density serum lipoprotein (Fig. 15-2). The same study indicated that the binding occurs exclusively to the hydrophobic domain of cytochrome b_5, the polar domain being incapable of binding simple amphiphiles in its native conformation. Other work (Holloway and Katz, 1975; Rogers and Strittmatter, 1975) has shown that cytochrome b_5 associates spontaneously with small phospholipid vesicles, with no perceptible change in vesicle size when the number of incorporated protein molecules is small, and only a moderate expansion when the protein content is as large as 230 protein molecules per vesicle. All these results are consistent with the simple mode of association described at the beginning of this chapter in which incorporation into a phospholipid bilayer is seen simply as one manifestation of a binding region designed to associate nonspecifically with any structure possessing a hydrocarbon–water interface, and to do so without greatly perturbing that structure.

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[1] In assessing the significance of this work, it has to be kept in mind that deoxycholate is not a typical amphiphile (p. 123) and its mode of association with proteins is not necessarily closely related to the binding of phospholipids or simple amphiphiles.

Binding studies of this kind have not been carried out with other intrinsic membrane proteins, but amphiphile-to-protein ratios at saturation of the protein have been measured in a few cases and are reasonably consistent with the concept of micellar binding. For example, monomeric bacteriorhodopsin (Reynolds and Stoeckenius, 1977) binds only slightly more Triton X-100 than cytochrome b_5, despite the fact that the hydrophobic binding region (presumably the entire external surface of the structure in Fig. 17-4) is very much larger. Bovine rhodopsin has been shown to associate with almost exactly one micelle of nonionic polyoxyethylene amphiphiles (Osborn et al., 1978).

It should be noted, however, that rhodopsin binds another amphiphile, dodecyl dimethylamine oxide, in an amount corresponding to two normal micelles (Sardet et al., 1976). Since the rhodopsin polypeptide chain is known to have two or more distinct trans-membrane segments (Fung and Hubbell, 1978), it is conceivable that this amphiphile (which has a very polar head group and a very high critical micelle concentration) might separate the trans-membrane segments so as to create two independent binding regions. However, there is no evidence to suggest this, and the result obtained is at present best interpreted as indicating that the concept of simple comicellization as a *general* mode of association between intrinsic membrane proteins and model amphiphiles is likely to be an oversimplification.

SPECIFIC LIPID–PROTEIN INTERACTIONS

The discussion so far has viewed the association between intrinsic membrane proteins and a phospholipid bilayer as a nonspecific consequence of the presence of a hydrophobic surface region on the protein, the lipid being simply a solvent for the protein, with a hydrocarbon layer of appropriate dimensions. It is likely that some membrane proteins interact more specifically with membrane lipids. For example, unsaturated hydrocarbon chains may be required at some locations to fit the surface morphology of the protein, or sites designed for specific lipid head groups may be present at the interface between hydrophobic and hydrophilic protein domains. A trivial example is suggested by the sequence of glycophorin shown in Fig. 17-1. Immediately following the hydrophobic segment there is a sequence of six residues containing four postively charged side chains, which may serve to anchor the protein to lipid molecules with negatively charged head groups. The six-residue segment is toward the carboxy terminal end of the chain, which lies on the cytoplasmic side of the membrane (see below), where phosphatidylserine would be the most abundant negatively charged lipid (p. 196). The amino acid sequence of fd coat protein has a similarly located segment rich in positively charged side chains, and it has been found that it can be readily solubilized by anionic but not by cationic detergents.

Qualitative evidence for lipid specificity exists for some membrane-bound enzymes on the basis of lipid requirements for expression of enzymatic activity. One of the earliest and best examples is provided by the mitochondrial enzyme β-hydroxybutyrate dehydrogenase (Sekuzu et al., 1963; Fleischer et al., 1966). This enzyme can be solubilized and partially purified by use of the bile salt anion cholate and by other means, but the solubilized enzyme has no catalytic activity. Incubation with suspensions of phosphatidylcholine from natural sources restores activity, but lipids with other head groups are ineffective. The requirement for a cholinelike head group is confirmed by studies with a series of lipid analogs (Grover et al., 1975), and the overall inference is that this protein associates specifically with phosphatidylcholine in the native membrane.

ASYMMETRIC INSERTION

As was noted at the beginning of this chapter, there is every reason to believe that the association of membrane protein with phospholipid in bilayer form is a *thermodynamic* phenomenon, dictated by an appropriately designed binding site on the protein molecule. This, however, raises a question for proteins that traverse the membrane from one side to the other, for it means that highly charged regions of the protein have to be transferred from the site of synthesis on the cytoplasmic side across the permeability barrier of the bilayer's hydrocarbon core. This problem is overcome in reconstitution experiments by carrying out the experiment under conditions where the bilayer is continually being disrupted by sonication or by the existence of a dynamic equilibrium with small lipid-detergent micelles (Warren et al., 1974). No such stirring-up can occur in the *in vivo* situation.

This problem is not unique to membrane proteins, but applies equally to cells that synthesize proteins for secretion into the extracellular fluid. How do the proteins cross the membrane? The problem has been studied by Blobel and his co-workers (Blobel and Dobberstein, 1975; Katz et al., 1977), who have shown that the mechanism involves a special "signal sequence" at the amino terminus of a nascent polypeptide chain destined to cross the membrane, and have postulated a special protein in the membrane to recognize this sequence and to allow the polypeptide containing it to move across. The same mechanism is likely to apply to intrinsic membrane proteins that have a single polypeptide segment traversing the membrane (e.g., glycophorin), with the difference that some additional feature of the mechanism halts the translocation process when the hydrophobic segment of the polypeptide has reached the bilayer core. At the present time, however, it is not easy to see how the Blobel mechanism can be extended to polypeptides that cross the membrane several times, as bacteriorhodopsin does, for example. One of the proteins in this category, the erythrocyte anion transport protein, is reported to have the amino terminus of its polypeptide chain on the cytoplasmic side of the membrane (Drickamer, 1977; Steck et al., 1978), whereas it should be on the opposite side if the initial insertion of the nascent polypeptide occurs by means of the Blobel mechanism. The amino terminus is on the expected side in glycophorin (Fig. 17-1) and in rhodopsin.

The biosynthetic mechanism for insertion of proteins into membranes, whatever its exact nature may prove to be, invariably assures that the insertion is asymmetric. In reconstitution experiments, however, one would expect the protein to be inserted randomly, and there is experimental evidence for this in some systems. However, there are also reports of asymmetric reconstitution, which is a puzzling phenomenon when the reconstituted system consists of vesicles made from a single kind of phospholipid, so that asymmetric distribution of lipid head groups cannot occur.

If the reconstituted system involves small single-walled vesicles of the type described in Chapter 11, the vesicles will have a very small internal volume and asymmetric insertion may then occur for purely steric reasons. The calcium pump protein from sarcoplasmic reticulum, for example, has a very large portion of the molecule exposed to the aqueous medium on the noncytoplasmic side of the native membrane (e.g., Stewart and MacLennan, 1974). When reconstituted into large vesicles, the protein is randomly oriented (Packer et al., 1974), but in small vesicles a high proportion of the molecules is oriented uniquely, with the bulky portion on the outside (W. L. Dean, unpublished data). Asymmetric insertion of the coat protein of fd bacteriophage into large vesicles has been observed, but it was shown that the reconstitution procedure that was employed led initially to the formation of small vesicles, which then fused to form larger vesicles (Zwizinski and Wickner, 1977). Asymmetry would be expected to be preserved when fusion occurs.

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